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CHOLINE AS A MEMBER OF THE VITAMIN B₂ COMPLEX

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PLATES I AND 2

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The experimental rations used in the study of the vitamin B₂ complex especially of vitamin B₂ and its related factors, are, as a rule, low in content of choline. This is also true of the basal diet deficient in vitamin B₂ which one of us (1) has employed during the last seven years in the production of rat acrodermatitis. It consists of 18 parts of casein, 68 of sucrose or rice starch, 8 of melted butter fat, 1 of salt mixture, 2 of cod liver oil, and is supplemented with thiamine and riboflavin. This diet is deficient in choline, but, until recently, no pathological changes attributable to this defect have been recognized in animals fed this mixture. On the whole, the ration appeared to serve its purpose without any visible interference due to the absence of choline.

Oleson, Bird, Ilvehjem and Hart (2) have lately reported the results of experiments in which choline (300 to 500 mg. per kilo) was added to the basal diet used for the studies of the vitamin B₂ complex, but these authors did not mention the reason for this precautionary measure.

The experimental data presented here not only substantiate the need for the incorporation of choline in a synthetic ration for rats used in the study of the vitamin B₂ complex but they also support the important interrelationship between content of choline and the ratio cystine/methionine in the diet. Du Vigneaud, Chandler, Moyer and Keppel (3) have called attention to the close relationship of choline and methionine metabolism. At about the same time and independently, Griffith and Wade (4) have pointed out the importance of the ratio cystine/methionine together with a low choline content in the diet.

EXPERIMENTS

Our first observations pertaining to the recognition of choline as a member of the vitamin B₂ complex were purely accidental. With the isolation and, later, with the synthesis of vitamin B₂, it became possible to go one step

further in the systematic analysis of the vitamin B₂ complex, namely, to incorporate crystalline vitamin B₆ in the diet which heretofore had contained only thiamine and riboflavin as the constituents of the vitamin B complex

From the time that vitamin B₆, as the third factor of the vitamin B complex, was added, in doses of 10 to 100 micrograms daily, to the diet of rats that had just been weaned and that weighed 20 to 35 gm, a growing number of fatalities was noted between the 11th and 15th days of the experiment. Death was preceded by failure to gain weight or even by decline of weight, but by no other visible signs of a specific disease

Gross Examination

At autopsy, in the gross, the most obvious abnormalities were observed in the kidneys and liver. In most animals that died before the 15th day, both kidneys were large, with a purplish red surface color, apparently due to intracapsular and subcapsular hemorrhage. On section, the cortex was diffusely dark red, or there were alternating patches of dark red and yellow throughout the cortex, in contrast to the grayish yellow color of the medulla. The color of the surface and of the cross sections of the liver was, as a rule, a uniformly light yellow, suggesting a high content of fat.

Microscopic Examination

Microscopically, the kidney showed a striking picture which varied in both type and degree (See Fig 1). Between the fibers of connective tissue in the capsule and also beneath the capsule, there was a variable amount of recently extravasated blood. The most pronounced lesion of the parenchyma consisted of almost complete necrosis of the tubules of the cortex, without destruction of the architecture. In most kidneys the outline of the tubules was still recognizable. In the neighborhood of the necrotic tubules there was a variable amount of infiltration with polymorphonuclear leucocytes. This type of exudation was never great and was absent or only very slight in many of the kidneys. Within the lumen of some of the convoluted tubules, especially the distal ones, and within the loops of Henle, as well as in some of the collecting tubules, there was a variable amount of homogeneous, acidophilic material in the form of casts of the lumen. Even in those kidneys in which the cortex showed the greatest pathological change, some of the collecting tubules showed only this hyaline acidophilic material in the lumen, their lining epithelium showed no microscopic abnormality. A striking feature of the diffuse necrosis of the cortex was the intense hyperemia of the interstitial tissue between the tubules and surrounding the glomeruli. The hyperemia was so intense in the cortex of some of the kidneys that it had the appearance of hemorrhage, and imparted the dark red color to the tissue observed in the gross. Definitely recognizable extravasation of blood was unusual, however, except at the extreme periphery of the cortex of some kidneys in which the blood appeared to have infiltrated into the cortical tissue from the subcapsular hemorrhage and did not seem to have originated from capillaries within the cortex itself. Hemorrhage in the capsular space around

the glomerular tufts or within the tubules of either cortex or medulla was not observed. In some of the kidneys in which the cortex was practically completely necrotic, glomeruli showed some degenerative change characterized mainly by swelling of the cytoplasm of the surface epithelium. A few of the glomeruli were intensely hyperemic, but there was no actual necrosis of the glomeruli, and there was no sign of inflammation. There were no recognizable pathological changes in the wall of the blood vessels, either large or small, and in none of these was premortem thrombosis observed. In some of the kidneys there was diffuse necrosis of the cortex without hyperemia, but with hemorrhage into and under the renal capsule. In other kidneys there were patches of cortical necrosis, with or without hyperemia, alternating with patches of hydropic degeneration and cloudy swelling. In such kidneys also a variable number of the tubules, especially those in the region at the junction of cortex and medulla, contained hyaline material in the lumen. Several kidneys showed marked diffuse hydropic degeneration of most tubules in the cortex with only a relatively small number of necrotic ones interspersed among them and with hyperemia of the interstitial tissue.

Microscopic examination of the liver showed the typical picture of marked diffuse fat infiltration (see Fig. 2) the cytoplasm of almost all the cells in the lobules being occupied by one vacuole or by a few large vacuoles. Occasionally a liver showed only a patchy lobular distribution of the fat or a moderate diffuse deposit limited to the periphery of the lobules.

Of 175 rats fed, from the first day after they were weaned, the experimental diet supplemented not only with thiamine and riboflavin (20 micrograms daily of each) but also with vitamin B₆ in an amount varying from 10 to 100 micrograms daily, 17 died between the 11th and 15th experimental days. 2 of these rats died on the 11th, 3 on the 12th, 3 on the 13th, 7 on the 14th and 2 on the 15th day. At autopsy, all showed typical capsular hemorrhage and cortical necrosis of the kidneys. The livers were light yellow and large in the gross.

Of this group of 175 rats, 5 more died in the further course of the experiment. Rat 32-00 died on the 17th day, rat 30-25 on the 23rd, rat 45-27 on the 24th day of the experiment, rat 26-13 after 4 weeks, and finally, rat 30-76 after 11 weeks. In the gross, these kidneys were characterized by irregular yellow patches with occasionally, small foci of hemorrhage under the renal capsule. In several of the kidneys the surface was roughly nodular and the kidney almost invariably was smaller than natural. Microscopically (see Fig. 3) there was a small amount of blood pigment within the capsule of some of the kidneys but there was little or no recently extravasated blood within or under the capsule, and many kidneys showed no sign of previous hemorrhage within or beneath the capsule. Most of the kidneys showed considerable shrinkage of the cortex, so that glomeruli, which are not naturally present immediately beneath the capsule, were closely crowded together in the periphery of the shrunken cortex, many immediately beneath the capsule. There appeared to be no reduction in the number of glomeruli but many of them were smaller than natural. There was little hyperemia in any portion of the kidney. In a few small foci in the cortex of some of the kidneys occasional tubules, mainly proximal convoluted tubules, showed remnants of necrotic lining epithelium. In the cortex of some of the most atrophic kidneys there was at least a relative increase of interstitial fibrous connective tissue. In the capsule of a few kidneys there was slight infiltration by lymphocytes. In the intermediate zone between cortex and medulla, a number of the tubules, mainly distal convoluted tubules

distinct signs of progressive recovery and healing. These rats often exhibited symptoms of sickness, such as anorexia and loss in weight, but they were definitely improving before they were killed. These observations are in accord with those of Cox, Smythe and Fishback (7), who also described recovery in rats receiving a diet high in content of cystine without any change in the experimental ration.

The effect of administration of 2 mg of choline daily was uniform in all rats, whether they received cystine or not. With the exception of one rat

TABLE I

Group	Supplement to basal diet	No of rats	No of rats that died before 16th experimental day	No of rats killed on 16th day	No of rats with renal lesions		No of rats with fat infiltration of liver				
					A- cute	Sub acute	++++	+++	++	+	-
1	Thiamine and riboflavin	21	0	21	0	1	12	5	3	0	1
2	Thiamine, riboflavin and cystine	14	4	10	5	5	12	1	1	0	0
3	Thiamine, riboflavin, cystine and choline	14	0	14	0	1	8	0	1	4	1
4	Thiamine, riboflavin and vitamin B ₆	32	2	30	5	5	27	1	2	0	1*
5	Thiamine, riboflavin, vitamin B ₆ and choline	18	0	18	0	0	3	3	3	5	4
6	Thiamine, riboflavin, vitamin B ₆ and cystine	19	5	14	8	6	15	1	0	1	2
7	Thiamine, riboflavin, vitamin B ₆ , choline and cystine	17	0	17	0	0	5	5	4	2	1

* Liver of one other rat was not examined

in group 2 which showed subacute changes, the renal lesions did not develop in these animals. That vitamin B₆ has a definitely provocative effect on the production of the specific renal lesions again becomes manifest in comparison of groups 1 and 4 in Table I.

It has been mentioned that in addition to the renal lesions, fat infiltration of the liver was a regular finding in all the groups that did not receive choline. Choline in the dosage used (2 mg daily) had a slightly or, in group 5, a definitely lipotropic effect, with the result that the histologically demonstrable fat infiltration was distinctly decreased or absent. Here

again the results are in favor of the assumption of a deficiency of choline in rats kept on basal rations commonly used in the study of the vitamin B₂ complex. Griffith and Wade (4) have shown that whereas 0.4 mg of choline per gm of diet regularly prevents renal lesions, the fat infiltration of the liver can be prevented or cured only by higher doses (2 mg of choline per gm of the experimental diet).

Apart from the renal lesions and fat infiltration of the liver, of the group of young rats fed the basal diet supplemented by thiamine, riboflavin and vitamin B₆, in 4 rats there developed severe flaccid paralysis of the hind legs, such as has been described by Sure (9) and attributed by this author to deficiency of choline. In the group of rats that received choline, no similar neurological manifestations were encountered.

The addition of 2 mg of choline daily had no appreciable preventive effect either on suprarenal hemorrhage or on panmyelophthisis (10) in rats fed a diet deficient in vitamin B₆.¹ Furthermore, in only 2 rats was hemorrhagic necrosis of the suprarenal glands found associated with cortical necrosis of the kidneys, while patchy or diffuse necrosis of the liver (11) was not observed in any of the animals with cortical necrosis of the kidneys.

DISCUSSION

In rats kept on a diet containing 18 per cent of casein, 8 per cent of melted butter fat, 68 per cent of sucrose, 4 per cent of salt mixture and 2 per cent of cod liver oil, with a daily supplement of 20 micrograms each of thiamine and riboflavin, the only lesion which could be attributed to deficiency of choline was, as a rule, fat infiltration of the liver. The addition of vitamin B₆ (10 to 100 micrograms daily) was followed in about 15 per cent of the experimental rats by necrosis of the renal cortex, with other characteristic histological findings.

The data presented in this communication serve to illustrate the importance of choline as a potential member of the vitamin B₂ complex and the aggravating influence of vitamin B₆ on the specific effect of choline deficiency on the kidney. Inasmuch as recently the determining factor for the need of choline has been found (4) in the ratio cystine/methionine

¹ In a more recent series of 169 rats fed the diet deficient in vitamin B₆ with the addition of larger amounts of choline (2 mg of choline per gm of diet) incidence of hemorrhage in the suprarenal glands, panmyelophthisis and hepatic injury remained practically unchanged.

² That choline may well be considered a member of the vitamin B complex has also been asserted by King (12, page 389) and by du Vigneaud, Chandler, Moyer and Keppel (3, page 75).

in the diet, the present experiments show that this quotient is by no means a fixed figure, but may be profoundly influenced by other conditions, such as content of vitamin B₆ in the diet. Griffith (13) found similar relationships to the absolute amount of cystine and methionine in the ration and to the nature of the fat added to the diet in the form of lard or cholesterol.

As to the character of the pathological changes in the kidney and the liver, it can be stated that they are identical with those caused by cystine intoxication (6, 7). In earlier and even in more recent investigations the renal lesions were produced by a diet containing casein, to which cystine was added, or by a diet containing proteins particularly rich in cystine, such as edestin or fibrin. In view of the fact that identical lesions develop in rats on a simple casein diet containing vitamin B₆ but without added cystine, these older studies now appear in a new light and need thorough reconsideration also from the point of view of practical implications.

The possible identity of the renal lesions in rats with bilateral symmetrical cortical necrosis of the kidneys which occurs in human beings, frequently in the pregnant female, should be mentioned. The nature and distribution of the cortical necrosis and the almost invariable bilateral involvement of the kidneys are suggestive of a similarity of the two conditions. There is, however, at least one definite and significant difference between the microscopic pictures of the two. Vascular thrombosis, which has been described in the human kidneys, and to which pathogenetic significance has been ascribed (14), was not observed in the kidneys of the animals. The pathogenesis of the renal lesions in the animals must therefore be attributed to a nephrotoxic effect acting directly on the parenchyma, and not to ischemia brought about by vascular thrombosis. Since thrombosis is not always found even in the human kidneys, and since it is sometimes not widely distributed in these kidneys, there remains the possibility that bilateral symmetrical cortical necrosis in human beings is also due to a nephrotoxic effect, with or without angiospasm, determined by a dietary deficiency, and that the thrombosis, as has been suggested by Ash (15), is only a terminal event that plays no primary part in the pathogenesis of the renal lesions.

SUMMARY

The experimental rations commonly used in studies on the vitamin B₂ complex are, as a rule, low in content of choline.

Addition of vitamin B₆ has an aggravating influence on the specific effect of deficiency of choline, especially with regard to the development of cortical necrosis of the kidneys.

The acute and subacute lesions associated with this specific type of renal injury are described.

The renal lesions and fat infiltration of the liver, observed in rats kept on a vitamin B free diet, supplemented with thiamine, riboflavin and vitamin B₆, are indistinguishable from those attributed hitherto to cystine intoxication

Attention is drawn to the rôle of choline as a potential member of the vitamin B₂ complex and, in this connection, to the importance of the ratio cystine/methionine in the diet

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EXPLANATION OF PLATES

PLATE 1

FIG 1 Rat 33-40 Fed diet deficient in vitamin B complex (including choline), with supplement of thiamine chloride, riboflavin and vitamin B₆

Kidney Acute diffuse necrosis and hydropic degeneration of tubules of cortex and intense hyperemia of the interstitial tissue There is hemorrhage into and beneath the renal capsule and some interstitial extravasation of blood in the periphery of the cortex Section stained with hematoxylin and eosin $\times 150$



(Gyorgy and Goldblatt: Choline as member of vitamin B₁₂ complex)

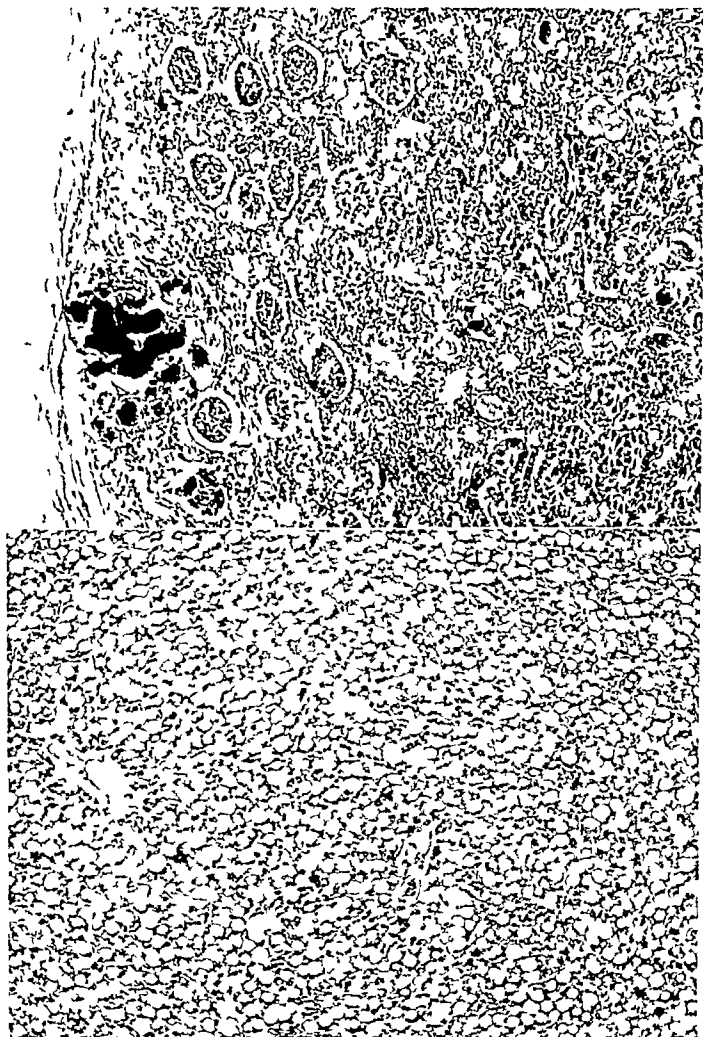
PLATE 2

FIG 2 Rat 45-66 Diet and supplements like those of rat in Fig 1

Liver Typical picture of marked diffuse fat infiltration Section stained with hematoxylin and eosin $\times 138$

FIG 3 Rat 31-08 Diet and supplements like those of rat in Fig 1

Kidney Subacute lesion Note remnants of necrosis and calcification of tubules, beginning fibrosis of cortex, and hyaline material in lumen of distal convoluted tubules and loops of Henle Hematoxylin and eosin $\times 138$



(György and Goldblatt: Choline as member of vitamin B₁₂ complex)

ELECTROPHORESIS OF THE COMPLEMENT FIXING ANTIGEN OF HUMAN INFLUENZA VIRUS

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The presence of antigens capable of fixing complement has been demonstrated in several virus diseases. In yellow fever (1), vaccinia (2), myxomatosis (3), and lymphocytic choriomeningitis (4, 5), for example, this antigen has been shown to be soluble and apparently can be freed from the virus itself, in others such as rabbit papilloma (6), it is so intimately associated with the virus that no distinction can be made between the two. The occurrence of a specific complement fixing antigen in mouse lungs infected with human influenza virus was reported by Smith (7) in 1936, and the work of Hoyle and Fairbrother (8) subsequently suggested that this antigen is soluble and separable from the virus.

Electrophoresis has contributed much to the newer knowledge of proteins and was therefore employed in the investigation reported here. It was applied to the study of the electrical mobility of the complement fixing antigen present in infected lung suspension, and also to the study of normal and influenza mouse serum. It was thus possible to give greater significance to the pH mobility curve of the antigen by comparing it with the curves obtained for the various components of mouse serum.

Material and Methods

Normal Mouse Serum—Normal albino Swiss mice about 1 month of age were bled from the heart under ether anesthesia. After being allowed to clot the blood was stored overnight at 4 C and on the following day the serum was removed after centrifugation. It was then dialyzed against the appropriate buffer for 2 or 3 days in the cold room and finally diluted 1:2 or 1:3 with the same buffer before being placed in the electrophoresis cell.

Influenza Mouse Serum, Acute Phase—Mice of the same age and breed were inoculated with the PR8 strain of virus, each receiving 0.05 cc. of a 0.1 per cent suspension of infected mouse lung intranasally under light ether anesthesia. Three to 4 days later, when severe pulmonary consolidation was known to have developed the animals were bled by cardiac puncture under ether anesthesia and the serum specimens were pooled. Dilution and dialysis were performed in the same manner as with normal serum.

Mouse Lung Suspension —Mice inoculated as just described were killed 3 to 4 days after inoculation. The lungs were removed, weighed, ground with alundum, and enough salt solution was added to make a 10 per cent suspension. Salt solution at different pH's was tried, but it was found that the amount of protein dissolved did not vary greatly. Therefore, in the experiments reported below either 0.85 per cent NaCl or a borate buffer of pH 9.8 was generally used.

After grinding, the suspension was centrifuged at 2500 R P M for 30 minutes and the supernatant drawn off and stored at -76°C . Just before use, further clarification was effected either by spinning the suspension in the open-air centrifuge of Bauer and Pickels (9) at 13,000 R P M for 30 minutes, or by filtration through asbestos. The final product, with a protein concentration of about 1.0 per cent, was perfectly clear. This clarity was retained during subsequent dialysis against alkaline buffers, dialysis against acid buffers, however, resulted in irreversible precipitation, which reached a maximum at pH 4.5 and below. (At pH 4.5 in acetate buffer, for example, the precipitate represented about one-third of the proteins present.)

Buffers —Serum and mouse lung suspensions were dialyzed in the cold room against various buffers. Acetic acid and sodium acetate buffers at pH 4.6, disodium phosphate and sodium acid phosphate buffers at pH 5.4, 6.0, 6.8, and 7.9, as well as boric acid and sodium hydroxide buffers at pH 9.8 were used. All buffers, except the last, had an ionic strength of 0.1. The borate buffer had an ionic strength of 0.04, as it was observed that in stronger concentration (0.1) this buffer caused splitting of the albumin peak.

Electrophoresis —In these experiments the apparatus originally described by Tiselius (10) with the medium size cell accommodating 11 cc. of solution was used. Photographs of the migrating boundaries were taken by the "schlieren scanning method" of Longworth *et al.* (11). In order to compensate for the migration of the boundaries during the passage of the current, the arrangement described by Longworth and MacInnes (12) was employed.

Throughout this paper the electrophoretic mobility (U cm², volt⁻¹, sec⁻¹) will be referred to simply as mobility. The mobility of the visible boundaries was determined by timing their displacement along a scale placed in the focal plane of the camera, or by measuring the distance separating them in the schlieren diagram from the location of the initial boundary before passage of the current. The mobility of the antigen was determined by sampling the fluid in the cell at various levels after the completion of a run, and testing the samples for specific activity. In detail, the latter procedure was as follows —

The cell was closed by sliding aside the two middle sections, the electrode vessels were disconnected and removed, and the cell was elevated in the water bath until one-half the top section was out of the water. The buffer in the top section was syphoned off, and the top and two middle sections were moved entirely to one side, leaving the bottom section to close the U tube. Then a long metal needle fitted to a 5 cc. syringe was slowly lowered by hand into the tube, and in general four samples per section, about 1.15 cm. in length and 0.9 cc. in volume, were successively removed. With a little care this could be accomplished without undue mixing.

Complement Fixation Test —The amount of antigen present in each sample removed after electrophoresis was determined by the use of the complement fixation test, original material which had not been subjected to electrophoresis serving as a standard for comparison. The test was conducted as follows. Serial twofold dilutions were made of each

specimen To 0.2 cc. amounts of each dilution were added 2 units of guinea pig complement followed by 0.2 cc. of a 1:10 dilution of inactivated pooled human influenza convalescent serum After incubation at 37°C for 1 hour, 0.5 cc. of sensitized sheep cells, consisting of a mixture of equal parts of a 5 per cent sheep cell suspension in saline and 2 units of amboceptor, was added Results were read after secondary incubation at 37°C for 30 minutes

This procedure was applied to lung suspension after electrophoresis at pH between 5 and 10 Electrophoresis of the antigen was not studied at pH below 5, since in that region it was found that the buffer was anticomplementary

EXPERIMENTAL

Electrophoresis of Normal Mouse Serum—Since schlieren patterns obtained from the ascending side of the U tube are usually easier to read than those from the descending side, only the former have been reproduced here Diagrams A and B of Fig. 1 give patterns obtained for normal serum at pH 6.8 and 9.8 Reading from left to right one goes from the top toward the bottom of the ascending side in the U tube Each peak represents one protein fraction On the basis of data obtained by Tiselius (13) for horse serum and by Stenhagen (14) for human serum, the principal peaks have been identified as albumin and as α and β globulins A small fraction, close to the albumin, has been labeled α The last peak, expressing a fraction which remained almost immobile, has been called $\gamma + \delta$, since it presumably includes Tiselius' γ globulin, and the "delta boundary disturbance," the latter is an anomaly, the origin of which has been analyzed by Longsworth and MacInnes (12), and is not due to an actual protein fraction This nomenclature has been used here as a matter of simple convenience There is little doubt that the fraction called albumin is nearly the same as the albumin fraction isolated by the usual precipitation procedures But no claim is made, however, as to the absolute identity of the α and β globulin fractions with the same fractions described by Tiselius or Stenhagen Diagrams A and B of Fig. 1 show complete similarity, except for the fact that the concentration in Diagram B is slightly greater, so that all the peaks are higher The mobilities of the various fractions, calculated from these diagrams, are given in Table I, which includes also the mobility of normal mouse serum albumin at pH 7.9

Electrophoresis of Serum from Mice Infected with Influenza Virus—Diagrams C, D, and E of Fig. 1 show the results obtained on a single specimen of pooled serum Diagrams C (pH 6.8, ionic strength 0.1) and D (pH 9.8, ionic strength 0.04) appear similar, the only difference being the appearance, at the higher pH, of a small new fraction labeled α' These patterns are similar to those obtained with normal serum under the same conditions

When a borate buffer of 0.1 ionic strength was used, as in Diagram E, the albumin was split in two, so that the α and α' fractions became invisible. The rest of the pattern remained unchanged. The splitting of the albumin suggests that this fraction is not as homogeneous as is usually supposed. The same phenomenon, at acid pH, has been observed by Luetscher (15) in horse and in human serum. Between Diagrams D and E the sample was dialyzed against acetate at pH 4.6, the slight precipitate formed was removed, and the sample was redialyzed against borate pH 9.8 and ionic strength 0.04. The pattern obtained then was exactly similar to Diagram D, except for the almost complete disappearance of the α globulin. This shows the slight effect on serum of acidification to pH 4.6, whereas the same procedure applied to lung suspension causes considerable precipitation.

TABLE I
Mobility of Mouse Serum Proteins (Anode)

Serum	Buffer	pH	Ionic strength	Mobility $\times 10^5$				
				Albumin	α	α'	α	β
Normal	Phosphate	6.8	0.1	5.5	5.0	—	3.5	2.3
	"	7.9	0.1	7.0	—	—	—	—
	Borate	9.8	0.04	10.2	9.5	—	6.6	5.2
Influenza	Phosphate	6.8	0.1	6.2	5.6	—	4.1	2.7
	Borate	9.8	0.1	8.7 7.5	—	—	5.9	4.4
	"	9.8	0.04	10.4	9.6	8.5	6.8	5.1
	After acidification to pH 4.6	9.8	0.04	10.0	9.1	8.2	—	5.0

The mobilities of the various serum fractions are given in Table I. As would be expected, the mobilities are much greater in the less concentrated borate. Comparison with the figures obtained for normal mouse serum shows that at pH 6.8 the various fractions in influenza serum had a slightly greater mobility. At pH 9.8 the mobilities are the same.

Electrophoresis of Lung Suspension. Optical Results.—Diagram F of Fig. 1 gives the schlieren pattern obtained at pH 9.8 after the original solution had been concentrated twice by pressure dialysis. The smaller boundary showed the same mobility as serum albumin and has therefore, for convenience' sake, been called albumin. The other boundary migrated like the hemoglobin which could be seen with the naked eye and has therefore been labeled hemoglobin. Never more than two boundaries were observed, and at other pH's they always showed the mobility that could

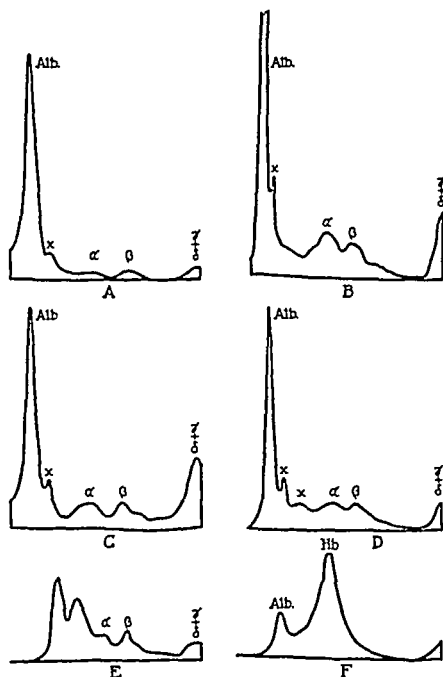


FIG 1 Electrophoretic diagrams of mouse serum and mouse lung suspension (anode)
 Abscissae distances measured from top of cell Ordinate concentration gradients

Diagram A Normal mouse serum pH 6.8, ionic strength 0.1 Dilution 1:3
 Potential gradient 8.8 V/cm After 111 minutes

Diagram B Normal mouse serum pH 9.8, ionic strength 0.04 Dilution 1:2
 Potential gradient 14.8 V/cm After 39 minutes

Diagram C Influenza serum pH 6.8, ionic strength 0.1 Dilution 1:2.5
 Potential gradient 8.7 V/cm After 100 minutes

Diagram D Influenza serum pH 9.8, ionic strength 0.04 Dilution 1:2.5
 Potential gradient 11.3 V/cm After 48 minutes

Diagram E Influenza serum pH 9.8, ionic strength 0.1 Dilution 1:2.5
 Potential gradient 7.4 V/cm After 72 minutes

Diagram F Influenza lung suspension pH 9.8, ionic strength 0.04 Potential
 gradient 12.2 V/cm After 24 minutes

summarized in Fig 2, in which the mobilities at 0.1 ionic strength have been plotted against pH. It is obvious that the complement-fixing antigen has a mobility between that of albumin and α globulin, or between the α fraction and α globulin, with an isoelectric point probably slightly above pH 5. At an ionic strength of 0.04 the relative position of the antigen is the same, as can be gathered from the mobilities in Tables I and II.

DISCUSSION

Presentation of the mobilities of serum protein fractions and complement-fixing antigen on the same chart (Fig 2) is obviously only a convenient device which makes the results more easily visualized. But we do not know whether the antigen is present at all in serum. It may be entirely absent from it, or present in amounts too small to be detected. It is probably not present in serum in large amounts, for, if some inhibiting factor made its detection there impossible, it would not be detected in the mouse lung suspension either, since the lung suspension contains so much blood.

The mobility of the complement-fixing antigen shows that it is electrophoretically similar to a serum globulin. Its mobility would be near that of α globulin on the alkaline side, but greater than α globulin at neutrality. More accurate conclusions are not warranted because of the crudeness of the sampling device and because of the experimental error in the complement fixation test, which make impossible an accurate determination of mobility.

Little is known of the physicochemical properties of the complement-fixing antigens of viruses. Hughes (1) found that the antigen present in yellow fever serum precipitated like an albumin with ammonium sulfate. Those present in vaccinia (16) and myxomatosis (17) precipitate with the globulins. In lymphocytic choriomeningitis the complement-fixing antigen is found both in the albumin and in the globulin fraction (5). One should bear in mind nevertheless that the present classification of proteins into "albumins" and "globulins," according to whether they precipitate at full saturation or half saturation with ammonium sulfate, has been derived from the study of normal serum. It may be extended to pathological sera or tissue proteins, as a matter of convenience, but only so long as its importance is not overestimated. That the soluble antigen of influenza virus stands in close relationship to the infectious moiety of the virus is suggested by preliminary experiments which indicate that the infectious agent has about the same electrical mobility as the soluble antigen, and also by the observation, which is at variance with Hoyle and Fairbrother's

conclusions (8), that it has not been possible to prepare a washed virus suspension entirely free from complement fixing properties. These points will be dealt with in later publications.

SUMMARY

1 An electrophoretic study has been made of normal mouse serum, influenza mouse serum, and influenza mouse lung suspension. The mobilities of the protein fractions present have been determined at various pH's by the optical method.

2 The pH mobility curve of the soluble (complement fixing) antigen present in the lung suspension has been determined analytically by sampling and application of the complement fixation test. The results show that the complement fixing antigen has a mobility definitely smaller than that of serum albumin and close to that of α globulin, with an isoelectric point close to pH 5.

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eliminate the repetition of the experiment in man, assuming that the proportions were similar in both instances. However, a method for extending the study of humoral immunity to malaria in man was suggested by the work of Knowles and Das Gupta (6) who succeeded in producing an infection with *P. knowlesi* in three human volunteers. Thus serum obtained from patients with induced *knowlesi* malaria could be tested for protective antibodies in *rhesus* monkeys. The same specimens could also be tested for the presence of agglutinins and complement-fixing antibodies. Accordingly, experiments were made using serum from patients with induced *knowlesi* malaria in an attempt to demonstrate the existence of humoral immune substances.

Methods

The source of the serum was one Negro patient at the New York Hospital¹ and six Negro patients at the Manhattan State Hospital,¹ all suffering from general paresis. Two had previously been found resistant to infection with *vivax* malaria when inoculated for therapeutic purposes. The patients were bled for serum and then inoculated intravenously or intramuscularly with 1 to 5 cc of citrated blood from a *rhesus* monkey after tests showed that the animal was suffering from no infection other than *P. knowlesi* malaria. The patients were then bled at various intervals after recovery from the acute attack.

Protection Tests—The protection tests with human serum were done by the method of Coggeshall and Kumm (7) with certain modifications aimed at increasing the sensitivity of the test. These modifications were the result of a previous study by Coggeshall and Eaton (8) which showed that the effectiveness of an immune serum was proportional to the number of parasites in the inoculum when incubated together 30 minutes at 37°C before injection. The test used in the present study consisted of mixing 2 cc of the immune serum with 10,000 *P. knowlesi* parasites, incubation at 37°C for 30 minutes, and the injection of the mixture intra-abdominally into normal *rhesus* monkeys. In certain instances there was no further administration of immune serum, while in others subsequent injections were made. In order to determine the protective property of any particular serum sample, either the amount of serum or the number of parasites in the inoculum was varied.

Complement Fixation Tests—The sera of six patients were tested for the presence and concentration of complement-fixing antibodies according to the method described by Eaton and Coggeshall (9), in which saline extracts of concentrated *P. knowlesi* parasites were used for antigens.

Agglutination Tests—Using the method of Eaton (10), attempts were made to determine the agglutinating titer of the same samples of serum used in the protection tests. The concentration of protective antibodies and agglutinins seemed to parallel each other, however, as the presence of heteroagglutinins seriously interfered with the tests.

¹ We wish to acknowledge the cooperation of Dr W. McDermott at the New York Hospital and Dr Ernest Kusch at the Manhattan State Hospital who were responsible for the management of these patients.

even after absorption, no definite conclusions could be drawn and the data are not included

EXPERIMENTAL

Protection Test 1—Protection tests were made on specimens of serum of patient W F who was inoculated intramuscularly with 2,000 000 *P. knowlesi* parasites, and the onset of clinical activity occurred 12 days later as evidenced by a fever of 38°C. There were six daily paroxysms and the acute disease terminated spontaneously. The maximum temperature during this period reached 39.4°C, and the greatest number of circulating parasites was 184 per 10 000 red blood cells. Serum specimens were obtained at the time of inoculation as well as 4 and 66 days after the acute disease had subsided. The protection tests were carried out with these specimens using 2 cc of serum and 10 000 parasites. The specimen obtained before infection and another

TABLE I
Results of Protection Tests with Specimens of Serum from Patient W F

Monkey No	Serum obtained after termination of acute attack	Additional serum injected		Result
		Amount		
		Daily	Total	
	days	cc	cc	
1	0	—	—	Died 14th day
2	4	—	—	No infection
3	4	2	20	Recovered
4	66	—	—	Recovered
5	66	2	20	Recovered
6	Normal human serum	2	20	Died 13th day

from a normal individual served as controls. Each of the two specimens of serum collected during convalescence was tested in two monkeys. One of each pair received only the 2 cc of immune serum in the inoculum while the duplicate received 2 cc of immune serum daily for 10 days.

The results, which are summarized in Table I, show that monkey 1, which received the preinfection serum specimen, and monkey 6, which received normal human serum, died on the 14th day and 13th day, respectively. Of the four remaining animals in which the convalescent sera were tested, monkey 2 developed no infection, while monkeys 3, 4, and 5 became infected but survived. The 20 cc of the 66-day serum given to monkey 5 in addition to that contained in the original inoculum apparently slightly diminished the intensity of the infection as compared with that of monkey 4. The results indicate that the serum from this patient had acquired protective antibodies in demonstrable concentration in the course of the induced infection.

Protection Test 2—Protection and complement fixation tests were made on the serum specimens of patient T H who had been inoculated intramuscularly with 3,500,000 *P knowlesi* parasites. The first sign of infection was a mild prodromal fever followed by a severe paroxysm and temperature of 40°C on the 11th day after inoculation. During the acute course of the disease which lasted 7 days there was a maximum temperature of 42°C, and the infection terminated spontaneously. The number of circulating parasites paralleled the fever curve, the greatest concentration being 306 parasitized cells per 10,000 normal red blood cells. The blood smears became negative soon after the temperature assumed a normal level and there were no relapses. A sample of serum was obtained before inoculation, at the height of the acute disease, and at 27 and 102 days after termination of the acute attack. These four specimens were tested simultaneously in individual normal monkeys for their protective effect.

The results are shown in Table II. It will be seen that no protective effect was demonstrable with specimens taken before inoculation, at the

TABLE II
Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient T H

Monkey No	Serum obtained after termination of acute attack	Result	Complement fixation titer
	days		
7	0	Died 11th day	0
8	At peak	Died 10th day	0
9	27	Died 9th day	1 1
10	102	Recovered	1 32

height of the acute attack, or in early convalescence. On the other hand, monkey 10, which received the serum obtained 102 days after the patient's recovery, survived the infection. The survival of this monkey further indicates the existence in the patient of a subclinical infection with the gradual accumulation of protective antibodies. An example of such an infection has been recorded by Milam and Coggeshall (11) where a patient after a spontaneous recovery from induced *knowlesi* malaria had normal temperatures and negative daily thick blood smears for 130 days, yet the blood was infectious for monkeys when subinoculated at that time.

The results of the complement fixation tests paralleled those of the protection test. The greatest concentration of complement-fixing antibodies was present in the serum that exerted the maximum protective ability.

Protection Test 3—Protection and complement fixation tests were made on the serum specimens of patient R G, who had been inoculated intravenously with 3,000,000 *P knowlesi* parasites. An elevation of temperature occurred 3 days later and was followed by six daily paroxysms with a maximum temperature of 41.2°C. The acute

disease terminated spontaneously and no relapses were noted. Serum was obtained before inoculation and 8, 28 and 56 days after clinical activity had subsided. The three specimens of serum taken from this patient during convalescence were tested in duplicate for their protective properties in six different monkeys, three received only the inoculum which consisted of 2 cc of immune serum and 10,000 parasites while the duplicates received an identical inoculum and an additional 1 cc daily of the same immune serum beginning the day that parasites were first detected in the blood smears. The results of these tests are shown in Table III.

It will be seen in Table III that a definite protective effect was obtained with the serum specimens obtained 8 and 28 days after the acute attack. There was no real evidence which showed that the injection of additional

TABLE III

Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient R G

Monkey No	Serum obtained after termination of acute attack	Additional serum injected		Result	Complement fixation titer
		Amount			
		Daily	Total		
	<i>days</i>	<i>cc</i>	<i>cc</i>		
11	0	—	—	No infection	—
12	8	—	—	Recovered	1 32
13	8	—	—	No infection	1 32
14	28	—	—	Died 16th day	1 6
15	28	1	8	Recovered	1 6
16	56	—	—	No infection	1 1
17	56	1	5 5	Died 12th day	1 1
18	Normal human serum	1	8	Died 11th day	—

serum had influenced the course of the infection in the test animals. The reason for the failure of monkeys 11, 13, and 16 to become infected is not known. It was observed that the serum from this patient agglutinated normal monkey red cells in extremely high dilutions, which may offer a possible explanation. The complement fixation test, also shown in Table III, reveals that the maximum titer of complement fixing antibodies was obtained with the sample of serum obtained 8 days after the acute attack had subsided. The 8 day specimen also exhibited the greatest protective effect. Thus it was seen that this patient had a definite serological immune response following the attack of induced malaria.

Protection Test 4—As the serum obtained from patient R G on the 8th day after the acute infection had terminated had the greatest protective ability, it was decided to test this specimen further by determining the protective antibody titer against a

varying number of parasites. Accordingly the 2 cc samples of the serum were incubated with 1,000,000, 100,000, 10,000, and 1000 parasites, respectively, and each mixture was injected into individual monkeys. One control monkey received 2 cc of normal human serum and 10,000 parasites.

The results are shown in Table IV, and it was found that the length of disease increased as the number of parasites was decreased in the inoculum. As the amount of immune serum was kept constant, these results are taken to indicate a definite protective effect. The failure of monkey 22 to survive when the dose of parasites was only 1000 does not invalidate the general result.

TABLE IV

Titration of Protective Effect with 2 Cc Serum of Patient R G Taken 8 Days after Recovery from Acute Attack

Monkey No	No. of parasites in inoculum	Result
19	1,000,000	Died 11th day
20	100,000	Died 13th day
21	10,000	No infection
22	1,000	Died 15th day
23*	10,000	Died 11th day

* Normal human serum

Protection Test 5—Serum specimens were obtained for protection and complement fixation tests on patient B A who was inoculated intravenously with 1 cc of infected blood containing approximately 10,000,000 *P. knowlesi* parasites. Clinical activity of the infection was mild, 5 days in duration, and terminated spontaneously. The maximum parasite count was 30 per 10,000 red cells. Serum samples were taken on 5, 17, and 53 days, respectively, after the acute attack had subsided and were tested in the same manner as was the serum of Protection Test 3.

The results shown in Table V indicate that the serum of this patient, who experienced an abortive clinical attack, showed a slight protective effect. Supplementary serum injections into monkeys 25 and 29 were associated with more prolonged infections than were obtained in monkeys 24 and 28, which received no additional serum, and all of the test monkeys which became infected lived longer than the control which received normal human serum. The maximum complement fixation titer was 1:8, which is a relatively low level.

Protection Test 6—In order to obtain specimens of serum for protection and complement fixation tests, patient T M was inoculated intramuscularly with 1 cc of in-

fect blood containing 3,000,000 *P. knowlesi* parasites. The incubation period was 23 days. The acute disease lasted only 4 days and was extremely mild with a maximum parasite count of 3 per 10,000 red cells. The acute infection terminated spontaneously. A preinoculation serum specimen was obtained and also specimens 10 and 36 days after the patient's temperature had returned to a normal level. The serum was tested in

TABLE V

Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient B A

Monkey No	Serum obtained after termination of acute attack	Additional serum injected		Result	Complement fixation titer
		Amount			
		Daily	Total		
	<i>days</i>	<i>cc</i>	<i>cc</i>		
24	5	—	—	Died 11th day	1 8
25	5	1	6	Died 12th day	1 8
26	17	—	—	No infection	1 4
27	17	1	7	Died 14th day	1 4
28	53	—	—	Died 11th day	1 2
29	53	1	10	Died 14th day	1 2
30	Normal human serum	1	10	Died 10th day	—

TABLE VI

Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient T M

Monkey No	Serum obtained after termination of acute attack	Additional serum injected		Result	Complement fixation titer
		Amount			
		Daily	Total		
	<i>days</i>	<i>cc</i>	<i>cc</i>		
31	0	—	—	Died 11th day	0
32	10	1	9	Died 10th day	1 16
33	36	1	8 5	Died 13th day	1 4
34	Normal monkey serum	1	10	Died 10th day	—

monkeys 31, 32 and 33 as in Protection Test 3, and the results are summarized in Table VI.

The fact that monkey 33 had a milder infection and survived longer than monkeys 31 and 32 is indicative of a stronger protective effect of the serum obtained 36 days after the termination of the acute attack than that of the preinoculation sample or the one taken at the height of the disease. In this instance the serum with the maximum titer of complement fixing anti

bodies apparently did not coincide with that showing the greatest protective effect

Protection Test 7—Protection and complement fixation tests were made on the serum specimens of patient L B. The inoculation was made intravenously with 18,000,000 *P. knowlesi* parasites, and parasites were first noted in the blood smears 10 days later, also the day of clinical onset. The acute disease lasted 5 days and the maximum temperature was 40.8°C. Only one convalescent serum specimen was taken after the temperature had returned to a normal level. Since there was only a single postinfection sample of serum obtained 16 days after the acute attack had spontaneously subsided, its protective qualities were compared to the one taken before inoculation, and a normal monkey serum control was added. The infecting dose of parasites for the protection tests was reduced to 1000 in these tests.

The results in Table VII show that the incubation of 4 cc. of the patient's serum obtained before the malaria infection exerted no inhibitory effect on

TABLE VII

Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient L B

Monkey No	Serum obtained after termination of acute attack	Amount of serum incubated with 1000 parasites	Result	Complement fixation titer
	days	cc		
35	0	4	Died 11th day	0
36	16	4	Recovered	1 12
37	16	1	Died 14th day	1 12
38	Normal monkey serum	4	Died 11th day	0

the disease of monkey 35. The use of an equal amount of convalescent serum and 1000 parasites resulted in a mild infection and survival when injected into monkey 36. Monkey 37, which received 1 cc. of the same serum and 1000 parasites, had a prolonged infection and succumbed on the 14th day. The additional control monkey 38 had a typical *P. knowlesi* infection and died on the 11th day. Complement fixation tests showed a substantial titer of antibodies, 1 12 in the postinfection sample of serum.

Protection Test 8—As a check on previous experiments in which it had been noted that the sera of certain patients during convalescence had a greater protective effect than those of others, it was decided to combine the remaining portions of the stronger specimens into pool 1 and compare its inhibitory action with that of pool 2, which was a mixture of those sera showing little or no activity when used in a protection experiment. The sera from these pools were tested in the usual manner for the presence of protective antibodies, and in addition an attempt was made to determine whether or

not the incubation of the serum with the inoculating dose of parasites influenced the course of the infection in the test

The results of this experiment, summarized in Table VIII, showed that monkey 39 was able to recover from its infection with the aid of the serum from pool 1, which was the more potent of the two pools. It was also indicated that the incubation of the immune serum and the parasites was a deciding factor in the favorable outcome of the infection in monkey 39, although this cannot be given too much credit because monkey 40 de

TABLE VIII

Protection Tests with Pool 1 and Pool 2 Serum with and without Incubation with 1000 Plasmodium knowlesi Parasites

Monkey No	Serum incubated with parasites	Pool No	Result
39	Yes	1	Recovered
40	No	1	Died 13th day
41	Yes	2	Died 14th day
42	No	2	Died 14th day

veloped a severe hemoglobinuria, and this complication usually results fatally for the monkey

DISCUSSION

That protective antibodies are found in the serum of animals following recovery from an acute attack of malaria is no longer doubted. In addition to the demonstration of the existence of these immune substances, it has been consistently pointed out that their concentration is extremely low. Actually this conclusion may be unjustified. The protection tests against some bacteria and viruses, involving the use of mice in large numbers, have reached such a high degree of refinement that the potency of the immune serum can be measured quantitatively in terms of the number of infective or lethal doses of the specific agent neutralized by a given quantity of the serum. No such test is practical in malaria. In testing an intracellular parasite such as the malaria plasmodium against its antiserum, it is apparent that a situation different from that of bacteria or viruses prevails. Presumably, the parasite in the inoculum is protected by a cell membrane which prevents an intimate contact between the organism and the immune serum. This inference has considerable basis in fact, for example, Eaton (10) has shown that an immune serum will agglutinate the malaria parasite only in the mature stage and when the red cell membrane is presumably

destroyed, or at least very permeable. The same serum was ineffective against the young ring or ameboid forms of the parasite. Likewise, in unpublished studies, it has been found in this laboratory that an immune serum *in vitro* is more effective against an equal number of segmenting forms than against young rings, although the former multiply within a few minutes into approximately 16 times more parasites. This indicates a greater protective effect by reason of accessibility of the immune serum to the parasite. Such a situation would explain why the results of the malaria protection tests in birds and monkeys are frequently indefinite, also why so many of the earlier experiments in passive immunity were negative. The parasite may be shielded from the action of immune serum not only during its growth within the red cell, but likewise throughout its entire cycle of development. It may be held that the opportunity for union between antigen and antibody would occur at the time the parasite sporulates, but just where this phenomenon takes place in the host is not known. In all probability the parasite either rapidly invades a new red cell outside the freely circulating blood or is phagocytized by a macrophage. In intense *P. knowlesi* infections one may obtain more than 50 per cent of red cells infected with mature segmenting forms and find only an occasional free parasite even when the animal's blood is examined at short intervals throughout the entire period of sporulation. It seems probable that in blood smears most, if not all, of the free merozoites seen outside the red cell are the result of mechanical disruption. Furthermore, Eaton and Coggeshall (12) showed that an anti-monkey red cell serum lysed infected red cells and rendered the liberated parasites non-infectious. This indicates that the free malaria parasites within the peripheral blood stream are probably not associated with, or essential to, the natural course of infection.

Another indication that the parasite is protected from the action of an immune serum is that in chronic *P. knowlesi* infections one is able to find a rather constant number of circulating parasites in a serum that can be shown to possess a relatively high concentration of protective antibodies. The observations cited, as well as many others, may explain why the results of the malaria protection tests cannot be compared with those obtained in many other diseases. Therefore, it would seem more logical to assume that the protection tests with malaria immune serum as used are inadequate to demonstrate the actual titer of protective antibodies which may be present in a considerable concentration. From the same evidence it can be assumed that immunity in malaria is relatively ineffective and temporary, not because the immune substances are present in low concentration, but because they are unable to exert their maximum influence, either directly or indirectly, against the parasite.

The results of the complement fixation tests may be discussed briefly. In all instances the preinoculation samples of sera were negative, while the convalescent specimens contained varying concentrations of complement fixing substances. There was no uniform correlation between their titer and protective action of the serum. Although there is no evidence to indicate that complement fixing antibodies and protective antibodies are identical substances, they are both produced as a result of malaria infection.

In the preceding experiments it is to be noted that none of the patients experienced a clinical attack which persisted longer than 7 days. Also, all infections subsided without the aid of any treatment. This type of infection is commonly observed in Negroes, who have a marked resistance to *P. knowlesi* infections as compared with white persons. A more definite protective effect might have been obtained with immune serum taken from individuals more susceptible to this parasite.

SUMMARY

Evidence has been presented which indicates that antibodies appear in the serum of individuals following an induced malaria infection with *Plasmodium knowlesi*.

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STUDIES ON ANTIBACTERIAL IMMUNITY INDUCED BY ARTIFICIAL ANTIGENS

II IMMUNITY TO EXPERIMENTAL PNEUMOCOCCAL INFECTION WITH ANTIGENS CONTAINING SACCHARIDES OF SYNTHETIC ORIGIN

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The functional rôle of bacterial polysaccharides in immunity has been amply demonstrated in studies from this and other laboratories. Although knowledge of the precise chemical constitution of the bacterial polysaccharides is still meager, it has been possible in some measure to correlate their specific immunological properties with differences in chemical structure. Several years ago we demonstrated that type specific antibacterial immunity could be evoked in rabbits with an artificial antigen containing the azobenzyl ether of the capsular polysaccharide of Type III *Pneumococcus* (1). More recently it has been shown that an antigen containing the azobenzyl glycoside of cellobiuronic acid, the pattern unit of the Type III pneumococcus polysaccharide, evokes in rabbits antibodies which agglutinate Type III pneumococci and confers passive immunity on mice against infection not only with Type III but with Type II and VIII organisms as well (2).

Cellobiuronic acid (4- β glucuronosidoglucose) is an aldobionic acid constituted from a molecule of glucose linked in β glucuronosidic union with a molecule of glucuronic acid on the fourth carbon atom of the hexose (3). Whether this exact molecular configuration is essential for eliciting pneumococcal antibodies in experimental animals is the subject of the present communication. A second antigen has therefore been prepared, one containing an isomeric aldobionic acid, gentiobiuronic acid (6- β glucuronosidoglucose) (4). The immunological properties of the antigen containing the synthetic saccharide have been compared and correlated with those of the antigen containing cellobiuronic acid, the aldobionic acid derived from natural sources. Cellobiuronic and gentiobiuronic acids are isomers differing in the position but not the configuration of the β glucuronosidic union as can be seen from Figs 1 and 2.

in the reaction not established the direct participation of the enzyme in the transfer reaction, they did indicate an exchange reaction between

TABLE III

Reactions Involving Exchange between Hydrogen of DPNH or Acetylpyridine DPNH and Medium (D₂O) Catalyzed by Diaphorase

The reaction mixtures in all experiments contained 170 μ moles of phosphate buffer, pH 7.5, and, in addition, the following: Experiment 1, 59.8 μ moles of DPNH, 105.7 μ moles of DPN, and 12.5 mg of diaphorase protein; Experiment 2, 56.4 μ moles of DPNH, 0.05 ml of purified *Neurospora* DPNase, and 12.5 mg of diaphorase protein; Experiment 3, 126.8 μ moles of DPNH and 19 mg of diaphorase protein; Experiment 4, 38.1 μ moles of acetylpyridine DPNH and 12.5 mg of diaphorase protein. All reaction mixtures were diluted to a total volume of 10 ml with D₂O. Reactions were started with enzyme, and incubated at room temperature anaerobically in an evacuated Thunberg tube for 3.5 hours. The reaction mixture in Experiment 3 was incubated for 6 hours. After these periods of incubation, the pH was adjusted to 9.5 with KOH and the reaction mixtures were placed in a boiling water bath for 2 minutes. The denatured protein was removed by centrifugation, and barium acetate was added to the supernatant solutions. The resulting precipitates were discarded after centrifugation. 5 volumes of cold ethanol were added to the supernatant solutions containing the barium salts of DPNH or acetylpyridine DPNH, and the solutions kept at 0° for 1 hour. The precipitates were collected by centrifugation, dissolved in 20 ml of H₂O, and left at room temperature for 30 minutes in order to remove any exchangeable deuterium. 5 volumes of cold ethanol were added and the solutions kept at 0° for 30 minutes before centrifugation. The precipitates were then treated as described in Table I. In Experiments 2 and 3 the isolated DPND was diluted with carrier glycine before analyzing for deuterium.

Experiment No	Electron donor	Electron acceptor	D ₂ O content of medium*	Nucleotide isolated	Deuterium content in nucleotide isolated†	
					Atom per cent excess	Atom per molecule
1	DPNH	DPN	95	DPND	1.34	0.36
2	"	None	99.5	"	4.01	1.08
3	"	"	93.3	"	2.74	0.74
4	Acetylpyridine DPNH	"	95	Acetylpyridine DPND	1.74	0.48

* Calculated value

† The deuterium values in Experiments 2 and 3 have a higher degree of accuracy than those in Experiments 1 and 4 because the DPND isolated in the former experiments was diluted with glycine before analysis, and the values given are corrected for dilution. The other values are uncorrected for purity. 1 atom of deuterium per molecule corresponds to a value of 3.7 atom per cent excess for DPNH and 3.6 atom per cent excess for acetylpyridine DPNH.

DPNH or acetylpyridine DPNH and the medium. As can be seen (Table III, Experiment 1), when DPNH and DPN are used as electron donor and acceptor, respectively, the DPNH isolated contained 0.36 atom of D

per molecule Since it was not apparent from this experiment whether oxidized DPN was necessary for this exchange, Experiments 2 and 3 were performed In Experiment 2 *Neurospora* DPNase was added to cleave any DPN which might be formed in the course of the reaction Experiment 3 contained no DPNase The DPNH present at the end of the incubation period in Experiments 2 and 3, respectively, contained 1.08 and 0.74 atoms of D per molecule These results clearly demonstrate that the hydrogen of DPNH is exchangeable with the medium (D_2O) under these conditions The lower value of 0.74 in Experiment 3, which

TABLE IV

Oxidation of DPND Formed in Exchange Reaction by Acetaldehyde and Alcohol Dehydrogenase, and Isolation of Nicotinamide from Resultant DPNH

The reaction mixture contained 37.4 μ moles of DPND isolated in Experiment 3, Table III, 0.6 mmole of phosphate buffer, pH 7.5, 3.6 mmoles of acetaldehyde, and 0.1 ml of crystalline yeast alcohol dehydrogenase in a total volume of 5.7 ml The reaction was started with alcohol dehydrogenase and incubated at 37° for 30 minutes After complete oxidation of the DPND, 0.05 ml of purified *Neurospora* DPNase was added to the reaction mixture and incubated at 37° for 1 hour After this period of time, no DPN remained The reaction mixture was placed in a boiling water bath for 3 minutes and cooled, and the denatured protein removed by centrifugation The resultant supernatant solution was analyzed for nicotinamide and placed on a Dowex 1-formate column The nicotinamide was eluted with H_2O , diluted 14.4 times with carrier nicotinamide, isolated by crystallization from benzene, and analyzed for deuterium

Deuterium content in DPND		Deuterium content in nicotinamide after alcohol dehydrogenase and DPNase treatment*	
Atom per cent excess	Atom per molecule	Atom per cent excess	Atom per molecule
2.74	0.74	8.2	0.49

* Values corrected for dilution with carrier nicotinamide 1 atom of deuterium per molecule of nicotinamide corresponds to a value of 16.7 atom per cent excess

contained no DPNase, is presumably due to a larger initial concentration of DPNH These experiments indicate that the exchange reaction between the hydrogen of DPNH and the medium does not require the presence of oxidized DPN A similar exchange has been noted (Table III, Experiment 4) between acetylpyridine DPNH and the medium

It should be noted that the deuterium values obtained in Experiments 2 and 3 are not quantitatively comparable to those in Experiments 1 and 4 because of the difference in the accuracy of the measurements (see Table III, footnote)

Stereospecificity of Exchange Reaction—It was indicated earlier that the diaphorase-catalyzed electron transfer reaction between DPNH and acetylpyridine DPN appears to show a greater affinity for Side 2 of reduced DPN

It was of interest, therefore, to determine whether the enzyme also shows this specificity for the exchange reaction between DPNH and D_2O . The DPND isolated in Experiment 3, Table III, which contained 0.74 atom of D per molecule, was oxidized with acetaldehyde and yeast alcohol dehydrogenase. The DPN formed was treated with *Neurospora* DPNase and the resultant nicotinamide isolated as described in Table IV. As alcohol dehydrogenase acts on Side 1 of reduced DPN, then, if deuterium is found in the nicotinamide, it would indicate that the deuterium in the DPND is located on Side 2. As can be seen in Table IV, the nicotinamide isolated contained 0.49 atom of D per molecule as compared to 0.74 atom of D per molecule in the starting DPND. It is apparent therefore that 69 per cent of the DPND formed in the exchange reaction is in Form B. Here again, as in the transfer reaction, the enzyme appears to show a greater affinity for Side 2 of reduced DPN.

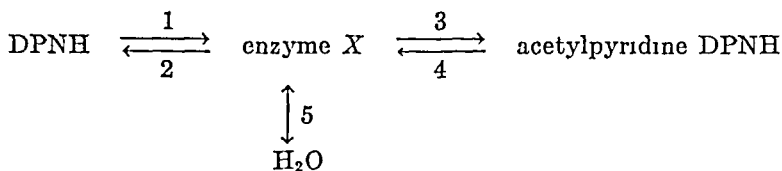
DISCUSSION

It has been possible with the use of D_2O to demonstrate that the mechanism of the transfer reaction between DPNH and acetylpyridine DPN, catalyzed by pig heart diaphorase, involves an electron rather than a direct hydrogen transfer. It was also shown that in addition to the transfer reaction there is an enzyme-catalyzed exchange reaction between the hydrogen of DPNH or acetylpyridine DPNH and the medium (D_2O). Although not indicated in Tables I to IV, no significant transfer or exchange was evident in the absence of the pig heart diaphorase.

Of interest are the data on the stereospecificity which indicate that the enzyme has an affinity for Side 2 of reduced DPN. This stereospecificity is the same for both the transfer and the exchange reactions. Of further interest is the fact that these results concerning stereospecificity show a similarity to the chemical reduction of DPN in D_2O with dithionite. In this non-enzymatic reduction the stereoisomers formed are approximately 70 per cent in Form A and 30 per cent in Form B. However, in both the transfer and the exchange reactions described here, it appears that the enzyme catalyzes the removal or exchange of hydrogen or deuterium from Form A at approximately 30 per cent of the rate of Form B.

No identification of the stereoisomer of acetylpyridine DPND, formed in both the transfer and exchange reactions, was possible, as it is not known whether yeast alcohol dehydrogenase catalyzes the removal of hydrogen or deuterium from the same side of acetylpyridine DPNH or acetylpyridine DPND as it does from DPNH or DPND. This determination, therefore, awaits data on the stereospecificity of the oxidation of acetylpyridine DPNH catalyzed by yeast alcohol dehydrogenase. The possibility that the observed stereospecificity is due to an isotope effect has been ruled out.

In view of the findings presented in this and previous papers (1, 2), it may be proposed that the electron-transferring system involves a series of reactions with (a) a direct transfer of an electron to a grouping on the enzyme forming a reduced protein, (b) release of H^+ to make a semireduced DPN, and (c) subsequent transfer of an electron to an acceptor. The following is a schematic representation of the reactions involved in the catalysis of transfer of electrons from DPNH to acetylpyridine DPN, where X refers to some reducible group on the enzyme



With D_2O in the medium, the following reactions were shown to occur. Reactions 1, 2, and 5 in the presence of enzyme and DPNH, and Reactions 3, 4, and 5 with enzyme and acetylpyridine DPNH. Reactions 1, 2, 3, 4, and 5 probably operate simultaneously when enzyme, DPNH, and acetylpyridine DPN are present. However, as mentioned previously (2), the enzyme does not catalyze the reduction of DPN when acetylpyridine DPNH acts as electron donor, presumably because of an unfavorable potential.

The rate of exchange of hydrogen between DPNH or acetylpyridine DPNH and H_2O in Reactions 1, 2, and 5 or in Reactions 3, 4, and 5 would be a function of the rate of transfer of electrons from DPNH to acetylpyridine DPN. Therefore, it would appear that the means of clarifying the mechanism of this transfer reaction would be to determine the rate of DPNH and acetylpyridine DPNH exchange with D_2O , and, in knowing the rate of formation of acetylpyridine DPNH from DPNH and acetylpyridine DPN, the reaction sequence could be ascertained.

The confirmation of an electron transfer mechanism in the reaction was indicated by the experiments cited in Table II. If a direct hydrogen transfer were involved, then the acetylpyridine DPNH isolated from the diaphorase-catalyzed transfer from DPND (Form A) or DPND (Form B) to acetylpyridine DPN would have been labeled with deuterium. As was shown, this was not the case.

The present results reinforce the earlier evidence that this transfer reaction is different from that catalyzed by the *Pseudomonas* transhydrogenase which catalyzes a stereospecific, direct hydrogen transfer (16).

Proof for an involvement of a reduced enzyme as the intermediate electron carrier is still lacking. However, the enzyme-catalyzed exchange reaction between DPNH or acetylpyridine DPNH and D_2O suggests that such a mechanism may be operating. Further experiments are still essen-

tial before FAD can be definitely ruled out as the immediate acceptor of electrons from the reduced coenzymes

SUMMARY

1 With deuterium as a tracer, the reduction of acetylpyridine DPN by DPNH, catalyzed by pig heart diaphorase, has been found to involve an electron rather than a direct hydrogen transfer mechanism. In addition, it has been shown that the enzyme also catalyzes an exchange between the hydrogen of DPNH or acetylpyridine DPNH and D_2O .

2 Although the evidence does not show absolute enzymatic stereospecificity, the enzyme appears to have some selectivity for Side 2 of reduced DPN in catalyzing the transfer and exchange reactions. This is the side opposite to that involved in the yeast alcohol dehydrogenase reaction.

3 Further evidence has been presented to prove that the enzyme is different from the *Pseudomonas* transhydrogenase, and a scheme for the mechanism of the reaction is presented.

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PRINCIPLES OF THE ENZYMATIC MEASUREMENT OF STEROIDS*

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Information on steroid concentrations in tissues is important for an understanding of the metabolism of these substances and also for diagnostic purposes in certain clinical conditions. Existing procedures for determining steroids, either singly or in mixtures, are based upon colorimetric methods or tedious bioassays. The merits and limitations of these procedures have been critically appraised (1-3), their principal drawbacks being lack of specificity and sensitivity. This paper concerns the microestimation of steroid hormones and their metabolites by enzymatic methods suitable for the measurement of these compounds in biological systems, and which appear to offer the promise of a high degree of sensitivity and specificity. Enzymatic steroid assays depend upon the quantitative interconversion of certain hydroxy- and ketosteroids by highly purified DPN¹-linked hydroxysteroid dehydrogenases prepared from *Pseudomonas testosteroni* (4-6). The accompanying changes in DPNH concentration are determined spectrophotometrically at 340 m μ (7) and constitute a stoichiometric measure of the amount of steroid oxidized or reduced. The enzymatic microestimation of steroids was demonstrated in 1953 (4) and its applications extended when more highly purified hydroxysteroid dehydrogenases became available (6). The enzymatic estimation of urinary steroids has been reported briefly (8). The present paper contains a detailed examination of the principles of the enzymatic measurements of steroids and considers their specificity, sensitivity, and accuracy.

At this time, two hydroxysteroid dehydrogenases are suitable for analytical purposes by virtue of their purity and high activity and a knowledge of their substrate specificities. The enzymes are highly active because of their inductive (adaptive) nature, and increases in specific activity of several 100-fold have been easily achieved by adding various steroids to the growth medium (6). The substrate specificities of these hydroxysteroid dehydrogenases have been described in detail (9, 10). In brief,

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† Scholar in Cancer Research of the American Cancer Society.

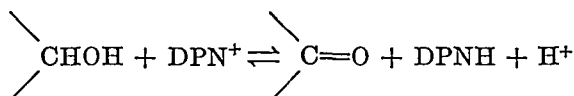
¹ The following abbreviations are used: DPN = diphosphopyridine nucleotide, DPN⁺ = oxidized diphosphopyridine nucleotide, DPNH = reduced diphosphopyridine nucleotide, DPNase = diphosphopyridine nucleotidase.

3 α -hydroxysteroid dehydrogenase (designated as α enzyme) catalyzes the reversible oxidation of 3 α -hydroxysteroids of the C₁₉, C₂₁, and C₂₄ series, whereas *3 β - and 17 β -hydroxysteroid dehydrogenase* (designated as β enzyme) reversibly interconverts 3 β - and 17 β -hydroxysteroids and the corresponding ketones. In order to employ these enzymes for the accurate estimation of steroids in urine, blood, and other tissues, a study of the factors influencing reaction equilibria was undertaken with a view to establishing conditions under which conversions are quantitative in the desired direction and under which interference from reaction products or inhibitors is negligible.

EXPERIMENTAL

Equilibrium Considerations

The reactions catalyzed by these pyridine nucleotide-linked dehydrogenases are of the general type



The oxidations of steroids are freely reversible and the equilibria may be displaced to the right (*forward-reaction*) or to the left (*back-reaction*) by employing mass action principles.

Forward-Reaction—The quantitative conversion of a steroid alcohol to ketone is favored by raising the pH of the reaction mixture, increasing the DPN⁺ concentration, and adding a ketone-binding reagent. The influence of pH on the velocity and equilibrium of the conversion of androsterone to androstane-3,17-dione by α enzyme is shown in Fig. 1. A high pH is advantageous in that the velocity of the oxidation is increased and equilibrium is attained more rapidly. Assurance of the displacement of the equilibrium to completion in the forward-reaction has been obtained by incorporating a ketone-binding reagent into the reaction mixture. Hydrazine is both convenient and effective in this respect, and its use is illustrated in Fig. 2, which shows the rate and equilibrium of the conversion of pregnane-3 α ,17 α ,21-triol-11,20-dione to its corresponding 3-ketone in the presence of varying concentrations of hydrazine at pH 7.8. Hydrazine is without significant influence on the initial reaction rate, but high concentrations of hydrazine permit the reaction to attain equilibrium more rapidly. By using hydrazine (0.3 M final concentration) in a reaction mixture of pH 9.5, advantage is taken of the greater efficiency of the reaction between hydrazine and ketones in basic solutions. Other ketone-combining reagents such as hydroxylamine (final concentration 1.0 M) and semicarbazide (final concentration 0.5 M) were also effective in shifting the

equilibrium, but these compounds inhibited the reaction rate of α enzyme slightly. Hydroxylamine is also undesirable because of its reaction with DPN^+ (11)

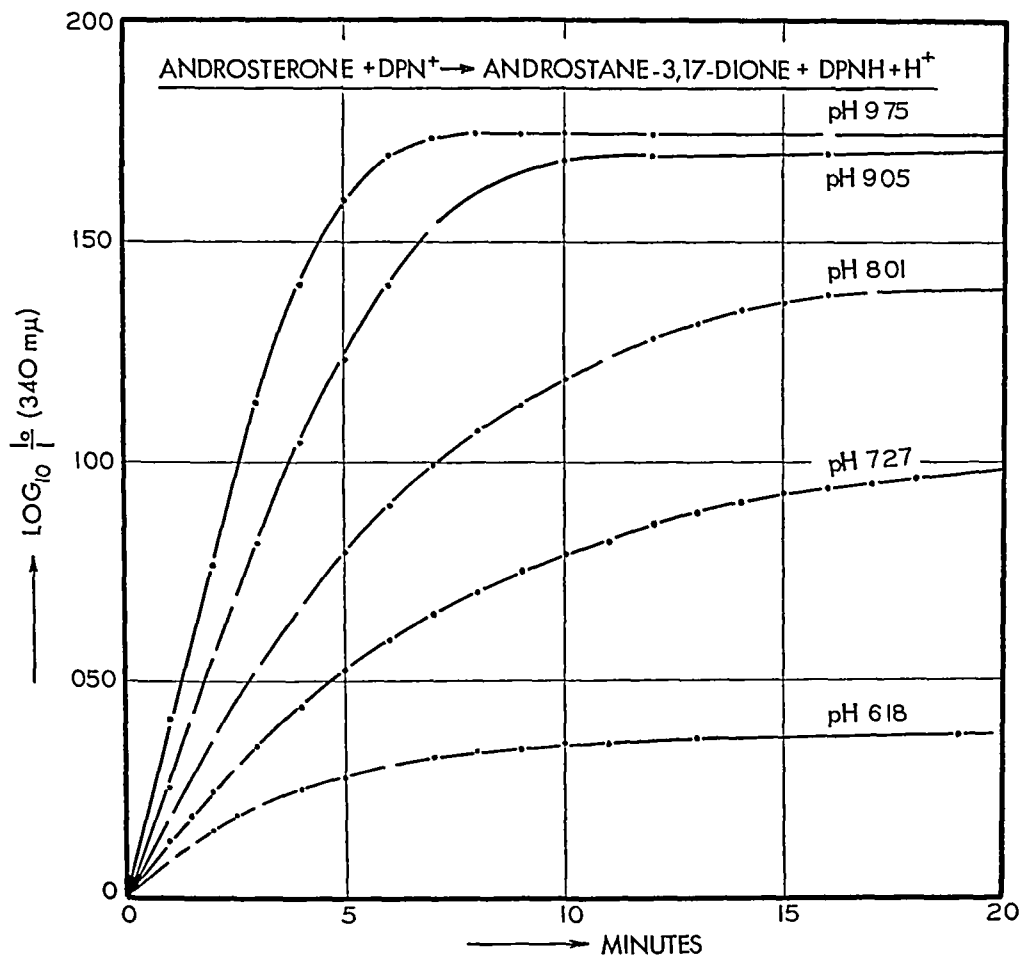


FIG 1 Time course of the oxidation of androsterone to androstane-3,17-dione by α enzyme at varying pH values. Reactions were carried out in 3.0 ml volumes in cuvettes containing 100 μ moles of Sorensen's phosphate (pH 6.18 to 8.01) or sodium pyrophosphate (pH 9.05 and 9.75) buffer, 0.5 μ mole of DPN^+ , 0.085 μ mole of androsterone in 0.1 ml of CH_3OH , and 33 units of α enzyme. Optical densities were measured at 340 $m\mu$ at intervals against blank cells containing no steroid. Temperature, 25°

Back-Reaction—The attainment of complete reductions of ketosteroids to steroid alcohols presents a more complicated problem. The equilibrium can be displaced in the desired direction by increasing the DPNH concentration and by lowering the pH (Table I). In practice, only limited use can be made of these factors, since DPNH is unstable in solutions more

acid than pH 5.5, and the concentration of DPNH must be kept quite low in order to permit accurate measurement of the decreases in optical density. However, for pure steroids the assays are satisfactory, as shown in Fig. 3, which demonstrates the equivalence of assays of epiandrosterone in forward- and back-reactions.

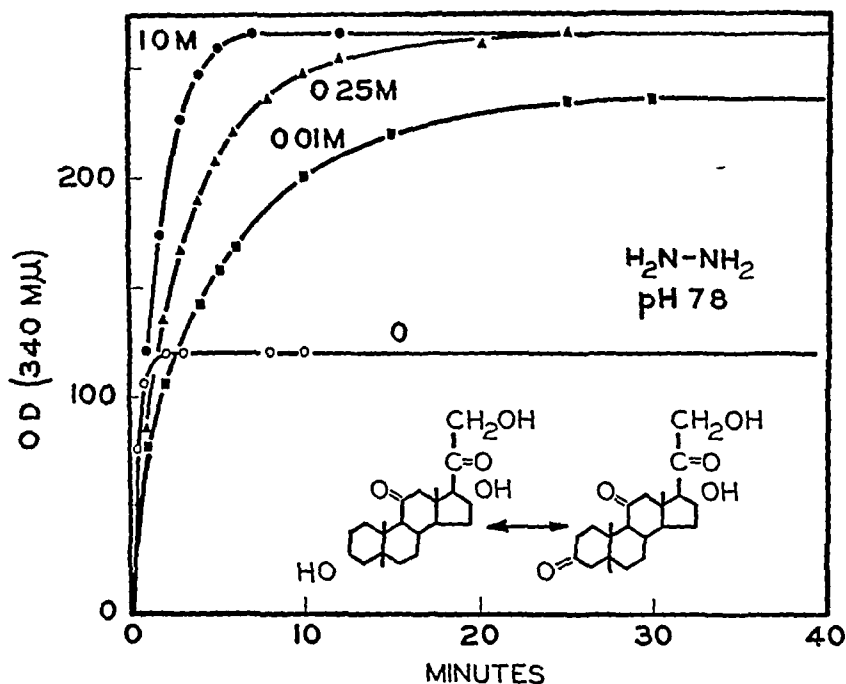


FIG. 2 Time course of the oxidation of pregnane-3 α ,17 α ,21-triol-11,20-dione by α enzyme in the presence of varying concentrations of hydrazine (0 to 1.0 M final concentration), as measured by the formation of DPNH at 340 m μ . The reactions were carried out in 3.0 ml. volumes containing 100 μ moles of Sorensen's phosphate buffer, pH 7.8, 0.5 μ mole of DPN $^{+}$, 0 to 3 mmoles of hydrazine, and 0.130 μ mole of steroid in 0.025 ml. of CH $_3$ OH. The reaction was initiated by the addition of 2150 units of α enzyme (specific activity, 47,000 units per mg. of protein). Temperature 25°.

Two methods for obtaining complete reductions in the back-reaction were investigated. By use of relatively large amounts of DPNH, the reduction of steroid was permitted to proceed to completion, the absorption of the excess DPNH at 340 m μ was destroyed by addition of HCl to pH 1, and the DPN $^{+}$ was measured by its complex with cyanide in alkaline solution (12). This gave satisfactory values with pure steroids, provided that the DPNH did not contain DPN $^{+}$. If the concentration of enzymatically prepared DPNH was raised sufficiently to drive the reaction to completion, there was interference from the α isomer (13) of DPN $^{+}$ which is present in enzymatically prepared DPNH, since the α isomer reacted with cyanide

but was not reduced during the enzymatic preparation. In the chemical reduction of DPN^+ with $\text{Na}_2\text{S}_2\text{O}_3$, both isomers react, but, in order to obtain a product suitable for measurement of small amounts of steroids

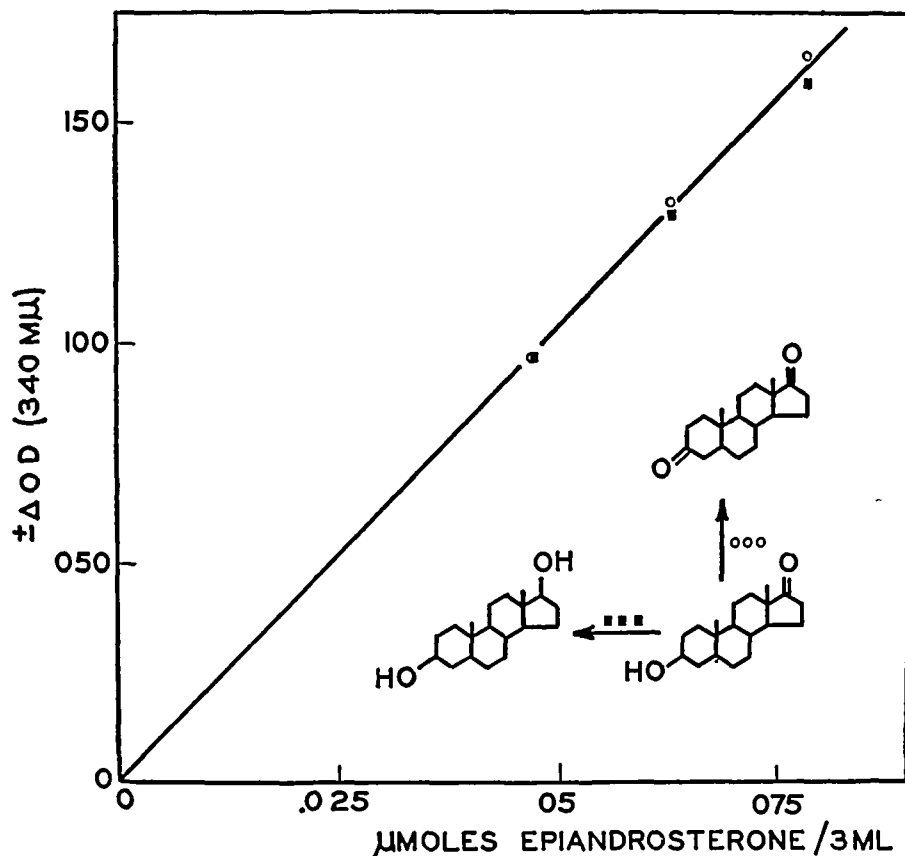


FIG 3 Enzymatic measurement of epiandrosterone with β enzyme by forward-reaction (O) and by back-reaction (■). Conditions for the forward-reaction were as follows: 100 μ moles of pyrophosphate buffer, pH 9.0, 0.5 μ mole of DPN^+ , appropriate amounts of epiandrosterone in 0.05 ml of CH_3OH , all in a total volume of 3.0 ml. Reactions were initiated by addition of 0.02 ml of β enzyme (118 units of specific activity 15,300 units per mg of protein). Increases in optical density at equilibrium were determined at 340 $m\mu$. Back-reactions were carried out in 3.0 ml volumes containing 100 μ moles of Sorensen's phosphate buffer, pH 5.5, 0.5 μ mole of chemically reduced DPNH , appropriate amounts of epiandrosterone in CH_3OH , and β enzyme as in the forward-reaction. Decreases in optical density (340 $m\mu$) were measured at equilibrium against a blank containing no steroid and set for an optical density of 0.250. Temperature 25°.

in the back-reaction by the cyanide complex, virtually all traces of DPN^+ must be eliminated, and this presents a goal not easily achieved in routine preparations.

An alternative method for attaining completion in the reduction of

steroid ketones is the removal of the oxidized DPN⁺ formed during the reaction by DPNase from zinc-deficient *Neurospora* (14). This enzyme selectively splits the nicotinamide-ribose linkage of the oxidized coenzyme only, and has been recently used to shift the equilibrium in the mannitol phosphate dehydrogenase system of *Escherichia coli* (15). *Neurospora* DPNase has a wide pH optimum, is relatively stable, and easily prepared. These considerations make the addition of DPNase a suitable and convenient method for attaining the desired equilibrium displacement. A comparison of the efficacy of *Neurospora* DPNase and pH changes in shift-

TABLE I
Effect of DPNase and Changes in pH on Equilibrium of Reduction of Androstane-3,17-dione to Androsterone by α Enzyme

pH	Equilibrium decrease in optical density (340 m μ)	Per cent completion of reduction
5.50	0.130	94.9
6.52	0.107	78.1
7.58	0.092	67.1
8.08	0.085	62.1
8.02 + DPNase	0.137	100

Reactions were carried out at 25° in 3.0 ml reaction volumes in 1 cm light path cuvettes containing 100 μ moles of Sorensen's phosphate buffer of indicated pH, 0.12 μ mole of chemically reduced DPNH (initial optical density approximately 0.250), 0.066 μ mole of androstane-3,17-dione in 0.05 ml of CH₃OH, and 1290 units of α enzyme (specific activity 47,000 units per mg of protein). Equilibrium was attained in 1 to 5 minutes (depending upon pH). 210 units of DPNase were added in the last instance. Readings were made against a blank containing no DPNH, and compensation was made for the slow spontaneous drop in optical density which the DPNH undergoes during the reaction time. All reactions were run in duplicate and mean values are given.

ing the equilibria is shown in Table I. The reduction of androstane-3,17-dione to androsterone was studied under the conditions indicated, at pH 8.08 the reduction was only 62.1 per cent complete, whereas the addition of DPNase shifted the equilibrium to completion, a condition not even attained by lowering the pH to 5.5. The advantages of using DPNase lie in that contamination of DPNH by DPN⁺ is not objectionable, and the concentration of DPNH may be low (about 0.03 μ mole per ml), thereby increasing the accuracy of the differential measurement.

Equilibrium Constants of Steroid Oxidations—The equilibrium constants involved in certain steroid oxidation-reductions have been measured with the aid of α and β enzymes.² The values are of some theoretical interest.

² Talalay, P., and Hurlock, B., unpublished observations.

in providing information on the energy differences between axial and equatorial substituents (16) The equilibrium constants (K_H) for these reactions have been defined as follows (17)

$$K_H = \frac{\left[\begin{array}{c} \diagup \\ \text{C=O} \\ \diagdown \end{array} \right] \left[\text{DPNH} \right] \left[\text{H}^+ \right]}{\left[\begin{array}{c} \diagup \\ \text{CHOH} \\ \diagdown \end{array} \right] \left[\text{DPN}^+ \right]}$$

Values of K_H for the oxidation of various equatorial 3-hydroxyl groups range from 0.9 to 2.1×10^{-9} and for axial 3-hydroxyl groups from 6.7 to 7.5×10^{-9} . The equilibrium constant for the oxidation of the "quasi"-equatorial 17 β -hydroxyl group of testosterone was found to be 37.8×10^{-9} . From these value ranges it may be calculated that, in a system containing initially 0.50 μ mole of DPN^+ , 0.10 μ mole of steroid, and with pH 9.5, a 17 β -hydroxysteroid will be 100 per cent oxidized. Under these conditions, an *axial* 3-hydroxysteroid will be 99.0 to 99.3 per cent oxidized, whereas an *equatorial* 3-hydroxysteroid will be 93.4 to 96.4 per cent oxidized. In the back-reaction, 0.05 μ mole of 17-ketosteroid in a reaction system containing 0.1 μ mole of DPNH at pH 6.0 will be 96 per cent reduced at equilibrium. These calculations assume no reaction product to be present initially, and do not include effects of hydrazine (forward-reaction) or DPNase (back-reaction).

Sensitivity, Specificity, and Applications

Sensitivity—The sensitivity of enzymatic assays is largely dictated by the spectrophotometric equipment, the physical dimensions of the cuvettes, and the reaction volumes employed. In routine measurements, which utilize 1.0 cm light path absorption cells, the volume may conveniently be varied from 0.2 ml to 3.0 ml. The smaller volumes require the use of microcuvettes (18). By assuming a desired optical density change of at least 0.030, the minimal quantity of steroid accurately measurable is 0.001 μ mole, or about 0.3 γ . Possibilities of increasing the sensitivity consist of either working in smaller reaction volumes (18, 19) or of using fluorescence measurements which can detect extremely small amounts of DPN^+ and DPNH (20). Fig. 4 shows the measurement of 0.2 to 1.0 γ of estradiol-3,17 β by oxidation to estrone with β enzyme.

Assay of Steroid Mixture—The estimation of single steroids in pure solutions has been amply illustrated in this paper as well as previously (4, 9). In order to examine the suitability of these enzymatic techniques for mixtures of steroids, solutions of four pure steroids in methanol were prepared. All compounds were of high purity, and their concentrations

were determined by α or β enzymes in the forward-reaction in at least two assays for each solution. The steroids used, and their concentrations by enzymatic assay, were as follows: androsterone 2.84 μ moles per ml, epiandrosterone 0.56 μ mole per ml, dehydroepiandrosterone 0.56 μ mole per ml, and pregnane-3 α ,17 α ,21-triol-11,20-dione 0.68 μ mole per ml. Accord-

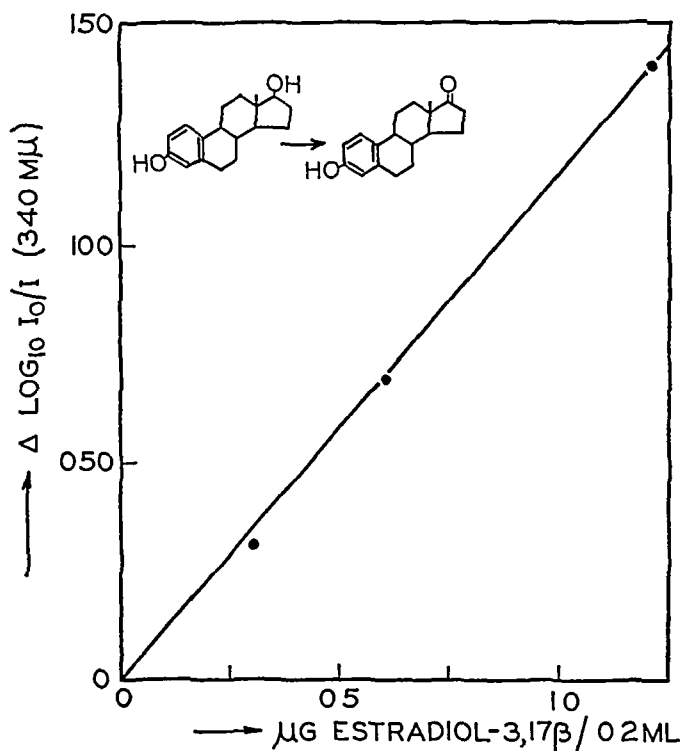


FIG 4 Estimation of estradiol-3,17 β by oxidation to estrone with β enzyme. Reactions were carried out in 1.0 cm light path microcuvettes in total volumes of 0.2 ml, containing 5 μ moles of sodium pyrophosphate buffer, pH 9.0, 0.05 μ mole of DPN $^{+}$, and varying amounts of estradiol-3,17 β in 0.02 ml of CH $_3$ OH. The reactions were initiated by the addition of 49 units of β enzyme (specific activity 24,400 units per mg of protein) in 0.005 ml. Optical density readings were taken at 340 $m\mu$ against blank cells containing all ingredients except steroid. Each point represents the mean of two measurements. Temperature 25 $^{\circ}$.

ing to these assays the steroids varied from 94.0 to 99.5 per cent in purity on the basis of weight. Equal volumes of the four steroid solutions were then mixed and aliquots of the mixture assayed at three concentrations in the forward-reaction, first α enzyme and then β enzyme being used. The 17-ketosteroids were determined separately with β enzyme by the cyanide complex-forming method. The assays are reproduced in Fig 5, which shows linearity with steroid concentration and reasonable agreement with calculated values. Table II gives the average values of the assays.

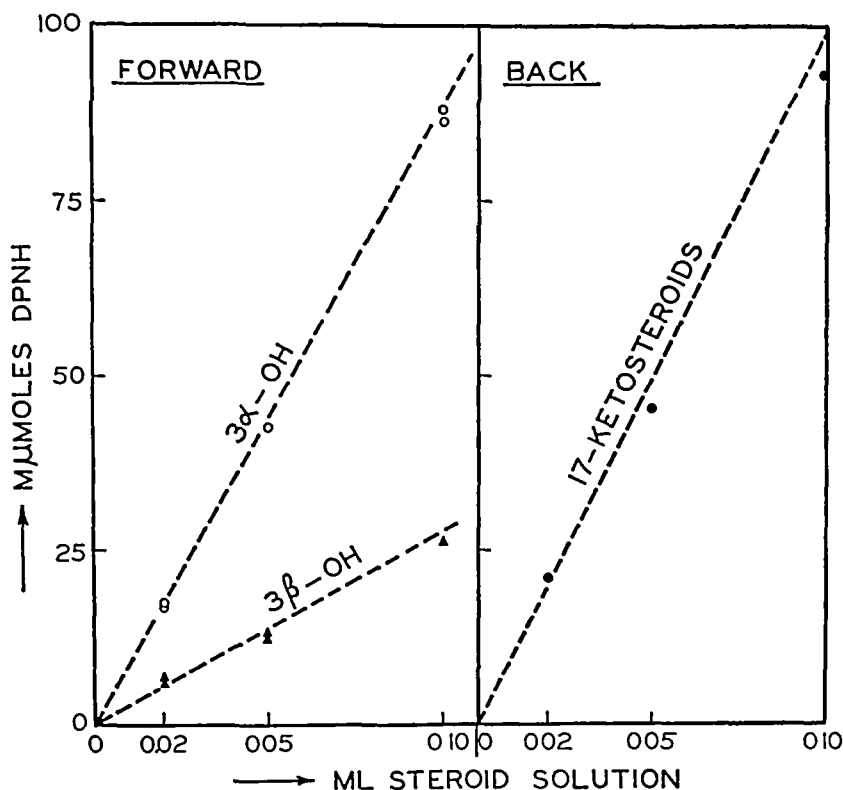


FIG 5 Analysis of a steroid solution prepared by mixing equal volumes of solutions of androsterone (2.84 μ mole per ml), epiandrosterone (0.56 μ mole per ml), dehydroepiandrosterone (0.56 μ mole per ml), and pregnane-3 α ,17 α ,21-triol-11,20-dione (0.68 μ mole per ml) in CH₃OH. The left graph demonstrates the amount of DPNH formed as a function of the volume of the steroid solution in the forward-reaction with α and β enzymes. The right graph is the amount of DPNH reoxidized in the back-reaction with β enzyme by varying volumes of the steroid solution. The lines are drawn so as to indicate the expected amounts according to the individual concentration of steroids in each solution before mixing. Temperature 25°.

TABLE II

Enzymatic Assay of Steroids in Mixture Containing Androsterone, Epiandrosterone, Dehydroepiandrosterone, and Pregnane-3 α ,17 α ,21-triol-11,20-dione in Methanol

Group assayed	Steroid per ml of mixture, μ mole		
	Found	Calculated*	Per cent discrepancy
3 α -Hydroxyl	860	890	-3.4
3 β -Hydroxyl	273	280	-2.5
17-Ketone	963	1000	-3.7

* Calculation is based on enzymatic assay of solutions of individual steroids before mixing. See the text for the design of the experiment.

and the amounts calculated. The assays of functional groups in the mixture were from 2.5 to 3.7 per cent below the calculated values.

Purity of Steroids—During the course of this work, a number of steroids with 3α -, 3β -, and 17β -hydroxyl groups were assayed by the appropriate enzymes, under conditions which assured complete oxidations. The compounds were believed to be of the highest purity, on the basis of preparative history, physical constants, elementary analyses, and infrared and ultraviolet absorption spectra, as well as in some cases by paper chromatography. These compounds were either prepared in our laboratory or obtained from other investigators. By enzymatic assay the purity of these compounds varied from 79.5 to 100.2 per cent according to weight. In many assays, a known amount of a steroid, pure by enzymatic assay, was added at the end of the reaction, and in each case the added steroid was assayed precisely in the presence of steroids of varying purity. Since the molar extinction coefficient of DPNH is known accurately from many independent measurements (21), enzymatic steroid measurements provide a convenient method for determining the purity of certain steroids and contamination by isomers. These determinations of purity are not dependent upon other steroids but merely upon accurate knowledge of the extinction coefficient of DPNH. Contamination of steroids by closely related compounds bearing similar functional groups cannot be detected by enzymatic measurement.

Analysis of Chromatographic Fractions—Enzymatic methods for the analysis of steroids have been useful for the estimation and identification of steroid fractions eluted from paper chromatograms or from chromatographic columns. The latter procedure is illustrated by the chromatographic separation of 400 γ each of 4-androstene-3,17-dione, dehydroepiandrosterone, and testosterone by gradient elution on a column of silicic acid (Mallinckrodt, $\text{SiO}_2 \cdot x\text{H}_2\text{O}$). A column measuring 103 mm. long by 11 mm. in diameter was packed from a slurry of silicic acid in hexane and chloroform (1:1 by volume), and the steroids were applied in a small volume of the same solvent mixture to the top of the column. A reservoir, 300 ml. capacity, provided with a magnetic stirrer was filled with this solvent mixture and connected to the column. The gradient of more polar solvents was established by introducing into the mixing flask methanol and chloroform (1:99 by volume). 5.0 ml. fractions were collected, evaporated to dryness, and redissolved in methanol, and aliquots were used for enzymatic analysis with β enzyme (for dehydroepiandrosterone and testosterone) and by ultraviolet absorption measurements (for 4-androstene-3,17-dione and testosterone) (Fig. 6). The total recoveries of all three steroids were 95 to 100 per cent, and the agreement between ultraviolet absorption and enzymatic analyses (for testosterone) was better than 5 per cent.

Specificity of Measurements—Since α and β enzymes carry out highly selective and stereospecific oxidation-reductions, it is possible to obtain much information on the qualitative and quantitative composition of steroid mixtures by appropriate sequential enzymatic oxidations and reductions. Thus, α enzyme in the forward-reaction measures total 3α -hydroxyl groups of the C_{19} , C_{21} , and C_{24} steroids. In the forward-reaction, β enzyme measures 3β -hydroxyl plus 17β -hydroxyl groups. In the back-

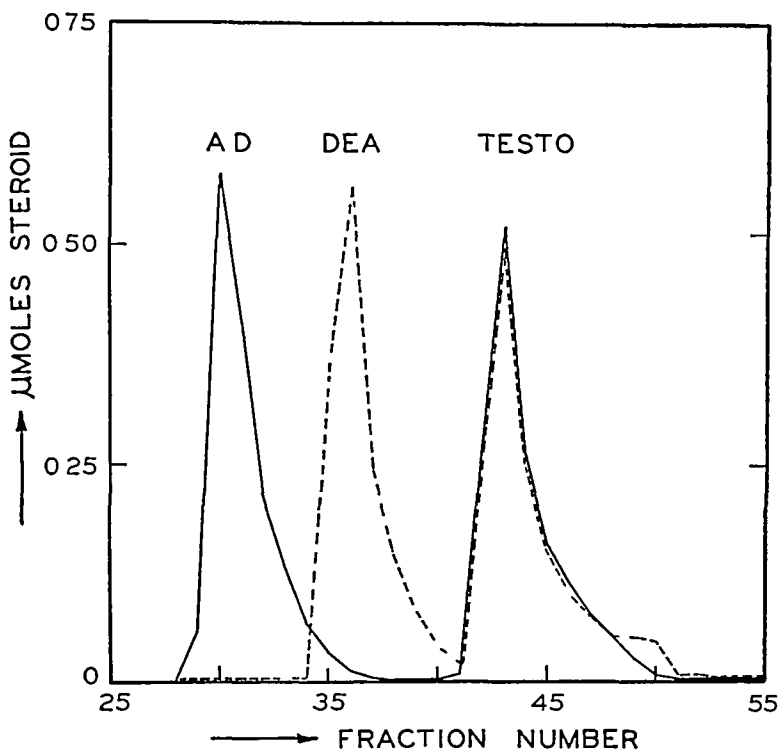


FIG 6 Chromatography of 4-androstene-3,17-dione (AD), dehydroepiandrosterone (DEA), and testosterone (TESTO) on silicic acid by gradient elution, as described in the text. The fractions (5 ml each) were analyzed by ultraviolet absorption (—) and enzymatic reaction with β enzyme (---).

reaction, α enzyme measures 3-ketosteroids only, whereas β enzyme reduces both 3- and 17-ketosteroids, provided that the ketonic groups are not in conjugation with a double bond. In the back-reaction, if α enzyme is used first and then followed by β enzyme, it becomes possible to obtain individual measurements of 3- and 17-ketosteroids.

In the forward-reaction, 3β - and 17β -hydroxyl groups are measured together, hence a $3\beta,17\beta$ -dihydroxysteroid will cause the formation of 2 equivalents of DPNH for each mole of steroid. Independent measurement of 3β -hydroxyl and 17β -hydroxyl groups may be obtained by measuring the 3-ketone groups with α enzyme in the back-reaction, before and after

oxidation of the steroid mixture with β enzyme, thus the increase in 3-ketosteroids will be equivalent to the 3β -hydroxysteroids originally present. Some of the functional groups which may be measured are listed in Table III. In addition, by the use of the very active steroid isomerase (22) which usually contaminates both α and β enzymes, it is possible to measure the specific increase in absorption at $248\text{ m}\mu$ (λ_{max} of α,β -unsaturated ketones in H_2O) and thereby obtain the increase in the α,β -unsaturated ketones following the oxidation of β,γ -unsaturated 3α - or 3β -hydroxysteroids. This measurement should be useful for measuring dehydroepiandrosterone (5-androsten- 3β -ol-17-one) and 5-pregnen- 3β -ol-20-one.

TABLE III

Groups determined	Reaction
3α -Hydroxyls	α enzyme, forward-reaction
3β - + 17β -hydroxyls	β enzyme, forward-reaction
3-ketones (<i>not</i> α,β -unsaturated)	α enzyme, back-reaction
3- + 17 -ketones (<i>not</i> α,β -unsaturated)	β enzyme, back-reaction
17 -Ketones	β enzyme in back-reaction, after α enzyme in back-reaction
3β -Hydroxyls	Measure 3-ketones with α enzyme before and after oxidation with β enzyme (3β -hydroxyls by difference)

DISCUSSION

The estimation of a variety of biochemical substrates by pyridine nucleotide-linked dehydrogenases has become a well recognized procedure. Enzymatic methods have proved especially suitable for steroid determinations for a number of reasons: (1) The substrate specificity of bacterial α - and β -hydroxysteroid dehydrogenases coincides with the most important steroid metabolites found in animal tissues. (2) The reactions catalyzed by these enzymes are readily reversible, and the equilibria can be easily displaced to completion in the desired direction. (3) The enzymes are saturated at very low steroid concentrations, and reasonable reaction velocities during the assays are maintained. (4) Enzymatic assay methods are highly specific, and the spectrophotometric methods are very sensitive. These last factors are indispensable in any analytical procedure requiring the measurement of minute quantities of specific metabolites in the presence of large quantities of a variety of other biological constituents.

In contrast to colorimetric methods based on chemical reactions such as the Zimmermann method for 17-ketosteroids (1), the enzymatic methods reported here offer several advantages in addition to those listed above. The measurements depend upon specific changes in light absorption at

340 m μ , under conditions which result in no unpredictable changes at other wave lengths. These changes in light absorption are, unlike those in many chemical methods, due to changes in concentration of DPNH, a well defined compound of known extinction coefficient and absorption maximum. In many chemical methods, the precise nature of the colored complex is unknown and there may be relatively large variations in extinction coefficient and maximal absorption wave length for different steroids. Thus, in the Zimmermann reaction, if 100 be taken as the relative extinction of dehydroepiandrosterone, equal amounts of other steroids give color equivalents from 65 to 130 (23). Moreover, the rate of formation of the colored complex depends upon many factors such as concentration of alcohol, alkali, length of color development, and temperature. Thus, in steroid mixtures, the total amount of color produced depends not only upon the amount of steroids, but also upon the proportion of different steroids and the conditions of color development.

The high degree of specificity of enzymatic reactions offers the advantage in many biological systems that unknown non-steroidal compounds which may be present are not likely to interfere. Thus, in the case of urinary steroid determinations, large non-specific "background" colors are produced when urine is treated with the Zimmermann reagents (KOH and *m*-dinitrobenzene). Many empirical attempts to correct these colors by differential spectrophotometry have not proved entirely satisfactory (1). In contrast, the addition of a few micrograms of enzyme protein to even a pigmented solution has been found not to alter the absorption spectrum appreciably in the region measured.

The sensitivity of these enzymatic methods approaches that required for the measurement of tissue concentrations of steroids. Enzymatic estimations with α and β enzymes permit the measurement not only of ketosteroids but also of hydroxysteroids for which no sensitive and specific methods have thus far been available. We visualize the discovery of further hydroxysteroid dehydrogenases specific for positions other than 3 and 17 on the steroid skeleton. Such enzymes may be of value in extending these methods for the determination of the quantitative and qualitative composition of steroid mixtures.

Methods and Materials

α - and β -hydroxysteroid dehydrogenases were prepared according to improvements of methods previously described (9). The addition of an acetone fractionation at -10 to -20° , after the first ammonium sulfate and protamine steps, has improved the purity of the enzymes so that enzymes of specific activities of 70,000 to 100,000 units per mg of protein have been obtained routinely before the calcium phosphate gel step.

Neurospora DPNase was prepared according to Kaplan (24) and had an activity of 52,000 units per ml, and 4.2 mg of protein per ml. The DPN⁺ was purchased from the Pabst Laboratories, Milwaukee, Wisconsin, and assayed 85 per cent DPN⁺ on a basis of weight (assuming a molecular weight of 663). DPNH was prepared either by reduction of DPN⁺ with alcohol dehydrogenase and ethanol (25) or by reduction with sodium hydrosulfite (26). Steroids were all checked as to identity and purity by melting point and optical rotation. Purifications of steroids were carried out whenever necessary. All the solvents were redistilled analytical reagent products. Hexane was purified by treatment with concentrated H₂SO₄ and alkaline KMnO₄ and distilled through a fractionating column. Hydrazine sulfate was Baker's analyzed reagent grade.

Assays of hydroxysteroids by the forward-reaction were carried out usually in 3.0 ml reaction volumes in cuvettes containing 100 μ moles of sodium pyrophosphate buffer (final pH 9.5), 0.5 μ mole of DPN⁺, 1.0 mmole of hydrazine sulfate (previously adjusted with NaOH to about pH 9.5), 0.01 to 0.1 μ mole of steroid in 0.1 ml of CH₃OH, and sufficient enzyme to achieve equilibrium in less than 15 minutes. Readings were taken against a control cuvette containing all ingredients except steroid. Measurements were based on the increments in optical density at 340 m μ at equilibrium. The molar extinction coefficient of DPNH at 340 m μ was assumed to be 6220 (21).

Assays of ketosteroids by the cyanide complex were carried out in 2.45 ml reaction volumes containing 100 μ moles of Sorensen's phosphate buffer, pH 5.5, approximately 0.1 μ mole of DPNH (chemically reduced), 0.01 to 0.10 ml of methanol solution of steroid, and sufficient enzyme to achieve equilibrium in 10 to 15 minutes. The initial optical density was about 0.250. Optical density measurements at 340 m μ were taken against a control cuvette containing all ingredients except steroid. When no further decrease in optical density occurred, 0.05 ml of 6 N HCl was added to control and experimental cells which destroyed all the absorption at 340 m μ . 0.5 ml of 5 N KCN in 1.0 N KOH was then added to all cells and the optical density again measured at 340 m μ . The assay was based on the increase in optical density upon addition of the cyanide. A molar extinction coefficient of 5150 was assumed for the DPN⁺-cyanide complex at 340 m μ (12).

Assays of ketosteroids in the back-reaction with DPNase were carried out in 3.0 ml reaction volumes containing 100 μ moles of Sorensen's phosphate buffer, pH 5.5, 0.1 μ mole of DPNH (chemically or enzymatically reduced), 200 units of *Neurospora* DPNase, 0.1 ml of methanol solution of steroid, and sufficient enzyme to obtain complete reduction in less than 15 minutes. Optical density readings were taken at 340 m μ against a

control containing no DPNH, an additional control containing all ingredients except steroid was also included. Measurements were based on the decrease in optical density in the experimental cell at equilibrium, compensated for small spontaneous reductions in optical density occurring in the control containing no steroid.

Ultraviolet absorption measurements were made in quartz cuvettes of 1.0 cm light path in a Beckman DU spectrophotometer. Microcells and a special carriage constructed according to Lowry and Bessey (18) were supplied by W. H. Kessel and Company, Chicago, Illinois.

SUMMARY

The sensitive and specific microestimation of steroids by means of α - and β -hydroxysteroid dehydrogenases has been described. Factors influencing the equilibria of the reactions catalyzed by these enzymes have been examined. Conditions for obtaining complete oxidations of hydroxysteroids or reductions of ketosteroids have been established. Quantitative oxidations of steroids have been obtained by using a high pH and adding a ketone-binding reagent. Complete reductions of ketosteroids have been achieved by the destruction with DPNase of DPN^+ formed in the reaction. The enzymes have been employed for the estimation of 3α -, 3β -, and 17β -hydroxysteroids as well as 3- and 17-ketosteroids, singly as well as in mixtures. The sensitivity of the method is about 1 μmole of steroid. The methods have been illustrated by the measurement of single steroids, steroid mixtures, and chromatographic fractions. These procedures are also applicable to the determination of steroid purity and contamination by isomers.

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THE EICOSAPOLYENOIC ACIDS OCCURRING IN THE GLYCEROPHOSPHATIDES OF BEEF LIVER*

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Polyunsaturated fatty acids occurring in animal tissues have been shown to be essential to living processes. Although many members of this group are known to exist in nature, differing by chain length and number and position of double bonds, few of the acids have been adequately characterized. Knowledge of the function and metabolism of these substances is meager, partly because methods of their detection and measurement are primitive. The best methods for their analysis lack standard values determined upon pure acids. Thus far, no preparations of these acids whose structures are known with certainty, and whose purities have been tested rigorously, have been adequately characterized by alkali isomerization. It is thus of importance to isolate pure acids of known structure and to utilize them as analytical standards in the existing methods of analysis for polyunsaturated fatty acids. This report describes some unsaturated acids of chain length of 20 carbon atoms which occur in the phosphatides of beef liver. The isolation and determination of structure of these acids were performed in the Institute of Physiological Chemistry of the University of Cologne, whereas the characterization of the acids by means of alkaline isomerization and paper chromatography was carried out at the Hormel Institute.

EXPERIMENTAL

The total lipides of beef liver were extracted with cold acetone and hot chloroform-methanol, and the phospholipides were isolated by precipitation with acetone from a concentrated solution in diethyl ether. The acids of the phospholipides were converted to methyl esters by acid methanolysis. The mixed esters were saponified with 0.5 N NaOH, the unsaponifiable matter was removed, and the freed fatty acids were frac-

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tionated by the low temperature crystallization method of Brown and Shinowara (1) From 141 kilos of phosphatides, 301 gm of highly unsaturated fatty acids were obtained, with an iodine value of 270 The methyl esters of these acids were fractionated by distillation at 10^{-4} mm pressure in a vacuum-jacketed column packed with small wire screen saddles made of V₂A stainless steel (2) Two distillations were required to obtain pure C₂₀ polyenoic esters, only those fractions which gave pure eico-

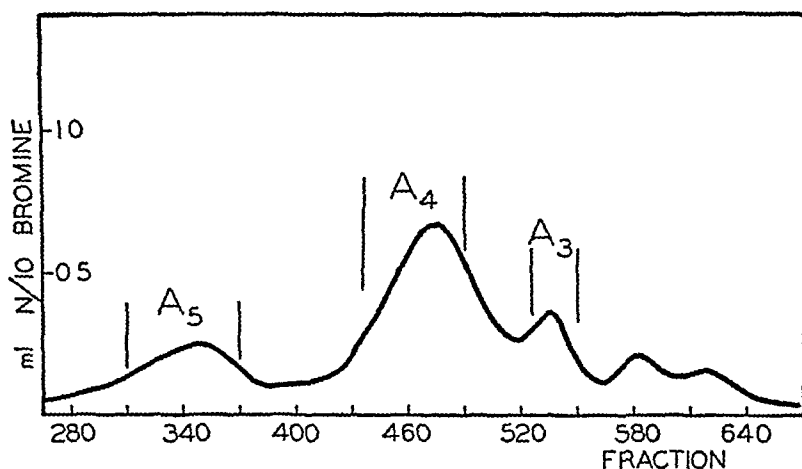


FIG 1

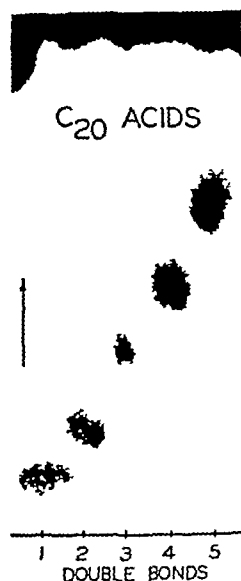


FIG 2

FIG 1 Separation of eicosapolyenoic acids by countercurrent distribution

FIG 2 Paper chromatography of pure eicosapolyenoic acids The mono- and dienoic acids were isolated from rape seed oil The spots on the developed chromatogram were made visible by iodine vapor and were contact-printed on Kodagraph paper, a procedure which gives a permanent record and increases the contrast between spot and background, thereby making detection of faint spots easier

sanoic acid after hydrogenation and saponification being retained The yield was 27 gm of esters, the fractions of which had iodine values of 282 to 292 The total quantity of C₂₀ acids present in the original mixture of highly unsaturated acids was several times this amount

The mixture of eicosapolyenoic acids was resolved into its individual components by means of a 200-stage fully automatic countercurrent distribution apparatus according to the method of Ahrens and Craig (3) The epiphase was *n*-heptane, and the hypophase was acetonitrile-acetic acid-methanol (1:1:1) Each stage contained 25 ml of epiphase and 25 ml of hypophase Epiphase was passed over the hypophase until the fractions no longer contained fatty acid For describing the partition curve, 2 ml portions of

every second or third fraction were taken for measurement of bromine uptake according to the method of Rosenmund and Kuhnhehn (4) (Fig 1) To find the limits of individual acid zones, single fractions were chosen according to the distribution curve and evaporated to dryness, and their ultraviolet absorption curves were determined qualitatively after isomerization (5) By means of spectral data, fractions were combined as shown in Fig 1 Composite Fraction A₅ was found to be hexaene-free pentaenoic acid, Fraction A₄ pentaene-free tetraenoic acid (arachidonic acid), and Fraction A₃ tetraene- and pentaene-free trienoic acid Each of these frac-

TABLE I
Characterization and Identification of Eicosapolyenoic Acids

	Fraction A ₅ (eicosapentaenoic acid)	Fraction A ₄ (eicosatetraenoic acid)	Fraction A ₃ (eicosatrienoic acid)
Iodine value, observed*	378	320	233
“ “ calculated	419	333 4	248 5
Equivalent weight saturated acid, observed, gm	312 5	312	312
Equivalent weight saturated acid, calculated, gm	312 4		
Melting point saturated acid, °C	74 2	74 4	73 8
Mixed melting point with <i>n</i> -eicosanoic acid (f p 73 1), °C	73 8	74	73 8
Double bonds indicated by isomerization	5	4	3
“ “ “ “ degradation (6)	5	4	3
Position double bonds indicated by degradation	5, 8, 11, 14, 17	5, 8, 11, 14	8, 11, 14, 5, 8, 11

* Iodine values obtained by the method of Rosenmund and Kuhnhehn are lower than those obtained by other methods

tions was contaminated with stopcock grease, which was easily removed as unsaponifiable matter From 3 82 gm of mixed eicosapolyenoic acids, 0 47 gm of pentaenoic acid (Fraction A₅), 1 39 gm of tetraenoic acid (Fraction A₄), and 0 39 gm of trienoic acid (Fraction A₃) were obtained It was assumed that Fractions 280 to 385 consisted of pentaenoic acid, Fractions 386 to 519 of tetraenoic acid, and Fractions 520 to 564 of trienoic acid The composition, calculated from the distribution curve and bromine uptake data, was thus eicosapentaenoic acid 16 per cent, eicosatetraenoic acid 53 per cent, eicosatrienoic acid 21 per cent, and unidentified substances 10 per cent

The unsaturated acids were degraded through ozonization Chromatographic analysis of the resulting dicarboxylic acids according to Klenk *et al* (6, 7) gave the following molar ratios glutaric acid to malonic acid,

0.81–2.44 for Fraction A₅, glutaric acid to malonic acid, 0.79–1.87 for Fraction A₄, and glutaric acid to suberic acid to malonic acid, 0.15–0.72–1.1 for Fraction A₃. As has been shown previously (8), the double bonds of these polyunsaturated acids are exclusively of the methylene-interrupted type (skipped unsaturation). The acids are, therefore, 5,8,11,14,17-eicosapentaenoic, 5,8,11,14-eicosatetraenoic (arachidonic), and a mixture of

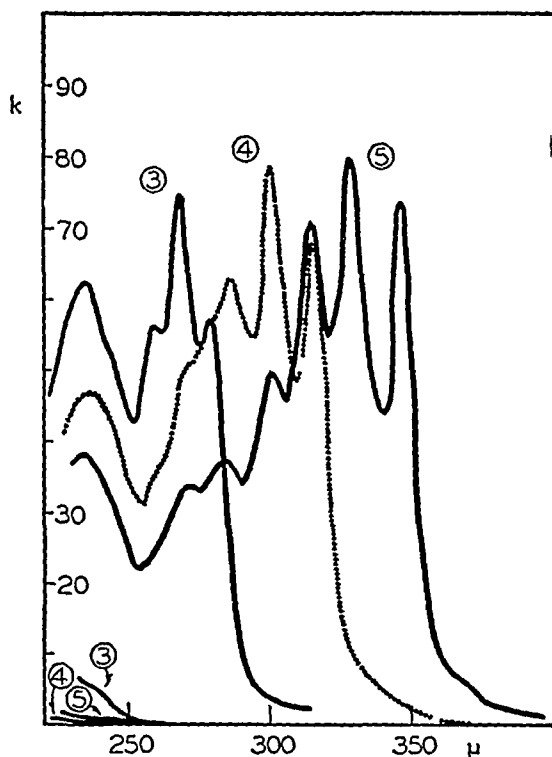


FIG. 3 Ultraviolet absorption spectra of eicosapolyenoic acids after isomerization according to the procedure of Herb and Riemenschneider (5). Curve 3, eicosatrienoic acid, Curve 4, eicosatetraenoic acid, Curve 5, eicosapentaenoic acid. The lower curves were obtained before isomerization, and the upper curves after isomerization.

8,11,14-eicosatrienoic and 5,8,11-eicosatrienoic acids. 5,8,11-Eicosatrienoic acid comprised about 10 to 20 per cent of the triene acids. The substances are all thin, clear, and slightly colored oils. The three acid preparations were chromatographed on paper by the method of Mangold *et al.* (9) and showed no evidence of contamination with acids of higher or lower degrees of unsaturation. A paper chromatogram on which these acids were run beside samples of eicosadienoic acid and eicosenoic acid from rape seed oil is shown in Fig. 2. This evidence, together with the spectral curves, indicates that the acids are of a high degree of purity. Characterization of these substances is summarized in Table I.

Approximately 30 to 50 mg of each of the purified fatty acids were dissolved in 10 ml of air-free ethanol, and the ultraviolet absorption spectra

TABLE II
*Extinction Coefficients, k , and Standard Deviations of Isomerized
Eicosapolyenoic Acids*

Method of isomerization	Wave length, $m\mu$									
	375	372.5	346	345	315	300	270	268	233	232.5
Eicosatrienoic acid										
Holman-Burr (10)*							52.8			57.5
							± 4.5			± 2.8
Wiese-Hansen (11)							37.4			55.4
							± 1.2			± 2.4
Herb-Riemenschneider (5)								72.1	59.5	
								± 0.4	± 0.2	
Holman (12)								87.0	56.7	
								± 1.6	± 2.3	
Eicosatetraenoic acid										
Holman-Burr (10)						61.9	62.8			52.1
						± 0.4	± 0.7			± 0.4
Wiese-Hansen (11)						48.9	48.1			62.1
						± 0.9	± 0.9			
Herb-Riemenschneider (5)					69.7			47.1	40.1	
					± 2.5			± 1.7	± 3.7	
Holman (12)					68.7			44.1	33.2	
					± 0.6			± 0.5	± 0.6	
Eicosapentaenoic acid										
Holman-Burr (10)		5.2		66.8		57.5	54.6			47.5
		± 0.5		± 4.4		± 1.4	± 2.5			± 1.6
Herb-Riemenschneider (5)	4.4		74.2		71.2			32.9	37.0	
	± 0.1		± 1.4		± 0.4			± 1.7	± 2.0	
Holman (12)	2.9		67.1		62.8			27.5	30.8	
	± 0.5		± 0.2		± 0.2			± 1.4	± 2.4	

* Bibliographic reference numbers

were measured upon these solutions. Aliquots of 1 ml were evaporated to dryness in petticups under nitrogen and were used as samples for isomerization according to the methods of Herb and Riemenschneider (5) and Holman and Burr (10). A portion was diluted 10-fold, and aliquots were isomerized by the methods of Wiese and Hansen (11) and Holman (12).

The complete spectra (Fig. 3) of the three acids after isomerization by

the procedure of Herb and Riemenschneider (5) indicate that each acid is free from its next more unsaturated vinylogue. The extinction coefficients of the three acids after isomerization by four methods are listed in Table II. The constants, which have been corrected for background absorption, can be used as standards for the analysis of these substances with the respective methods.

When the isomerization under the conditions of Holman (12) was continued for varying periods of time, the time for development of maximal characteristic absorption varied from acid to acid. The optimal time for eicosatrienoic acid isomerization is 20 minutes. The absorption *versus* time curve for arachidonic acid has high values for conjugated tetraene between 10 and 20 minutes. The absorption *versus* the time curve for eicosapentaenoic acid has a sharp apex at 12 minutes. These data are discussed and compared with those for other acids in the description of the method (12).

DISCUSSION

Recently a concept has developed that the polyunsaturated fatty acids occur in families related either to linoleic acid or to linolenic acid (13-15). If one counts the unsaturation from the methyl end of the fatty acid molecule, he finds that those acids which cure skin symptoms of essential fatty acid deficiency (for example, linoleic and arachidonic acids) have their first double bonds after the 6th carbon atom. The other polyunsaturated fatty acids have their first double bonds after the 3rd carbon atom, and these have only the growth-promoting activity. Similarly, the pentaenoic acid isolated in this study has the 3,6,9,12,15-pentaene structure which is related to the 3,6,9-unsaturated system of linolenic acid. This agrees with the observations that pentaenoic acid probably is derived from linolenic acid (16). The tetraenoic acid and the chief trienoic acid herein described have the terminal structure related to linoleic acid. Arachidonic acid is known to derive from linoleic acid (16, 17), and it is the most active essential fatty acid. In this study, the eicosatrienoic acid found in greatest abundance has a terminal structure related to linoleic acid, and probably is an intermediate in the conversion of linoleic acid to arachidonic acid. It could arise from linoleic acid by addition of one acetate group and by one dehydrogenation between the existing unsaturation and the carboxyl group.

The minor trienoic acid, 5, 8, 11-eicosatrienoic, has been previously isolated from the lipides of fat-deficient rats by Mead and Slaton (18). It is probably this trienoic acid which appears in the serum and tissues of a variety of animals when they become deficient in essential fatty acids. It appears that this substance is a normal minor constituent of liver lipides which merely increases during deficiency. Because its structure is not

similar to that of linoleic acid, and because it is found in deficient animals, it is probably inactive as an essential fatty acid. This acid has a terminal structure which relates it to oleic acid, for its first double bond, counting from the methyl end group, is at the 9th carbon atom. This suggests that the polyunsaturated acids may be divided into at least three groups.

The countercurrent distribution curve of the eicosapolyenoic acids suggests that eicosadienoic and eicosenoic acids are also present in the liver phosphatides of cattle, but positive identification of the two final small fractions obtained (Fig. 1) was not made.

The ultraviolet absorption spectra of the native acids indicate that they are notably free from preformed conjugation. Eicosatrienoic acid, the least pure in this regard, was estimated to contain less than 6 per cent conjugated diene. The spectra of the isomerized acids indicate that each acid is free from more highly unsaturated contaminants. The paper chromatogram confirmed this, and, moreover, showed no less unsaturated contaminants. The ultraviolet spectra of the isomerized acids indicated that the absorption maxima occur at the wave lengths reported by Herb and Riemenschneider (5). The wave lengths specified in the methods of Holman and Burr (10) and Wiese and Hansen (11) were chosen from the spectra of less pure materials. It appears that their measurement of tetraene at 300μ had the small advantage of less complication by the pentaene contribution. However, in the interest of standardization of methodology, Holman and Hayes (12) have made measurements at the same wave lengths as Herb and Riemenschneider. Our extinction coefficients determined upon tetraenoic acid with the method of Herb and Riemenschneider differ slightly from those reported by these authors upon their preparation. The conjugated tetraene absorption coefficient of our preparation was 69.7, whereas that of Herb and Riemenschneider was 60.6. Our eicosapentaenoic acid developed a conjugated pentaene absorption coefficient between those of the eicosapentaenoic and the docosapentaenoic acids of Herb and Riemenschneider.

SUMMARY

The eicosapolyenoic acids from beef liver phosphatides were isolated by low temperature crystallization of the acids and fractional distillation of their esters. The individual acids were isolated by means of countercurrent distribution. Ozonolysis and chromatographic separation of the degradation products of the tetraenoic and pentaenoic acids identified them as 5,8,11,14,17-eicosapentaenoic acid and 5,8,11,14-eicosatetraenoic acid (arachidonic acid). The triene consisted of a mixture of 8,11,14-eicosatrienoic acid and 5,8,11-eicosatrienoic acid.

The pentaenoic, tetraenoic, and trienoic acids were subjected to isomer-

ization according to four current analytical methods, and their extinction coefficients were determined as a means of characterization and as a means of standardization of the analytical methods. Each of the three acids was found to migrate as an entity when chromatographed on paper.

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KYNURENINE TRANSAMINASE OF RAT KIDNEY A STUDY OF COENZYME DISSOCIATION*

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Cell-free extracts of rat kidney catalyze the conversion of kynurenine to kynurenic acid by means of a transamination reaction (2). Such extracts lose kynurenine transaminase activity rapidly when added to phosphate buffer solutions with pH below 7 unless α -ketoglutarate is added (2). Further studies in this laboratory have shown that this inactivation is completely reversed by the addition of small amounts of pyridoxal phosphate or pyridoxamine phosphate. This observation suggests that inactivation results from dissociation of one or both of these coenzymes from the apotransaminase and that α -ketoglutarate prevents this dissociation. The present report seeks to describe this association-dissociation phenomenon and to explain the protective action of α -ketoglutarate.

Materials and Methods

L-Kynurenine sulfate monohydrate was obtained by ozonolysis of *N*-acetyl-L-tryptophan.¹ DL-Kynurenine, obtained commercially, was used in several experiments. DL-Kynurenine was less satisfactory as a substrate because the range of activity that could be measured spectrophotometrically was limited by absorption of the inert D isomer. Pyridoxal phosphate and pyridoxamine phosphate, each assayed as pure within the limits of accuracy of the spectrophotometric and chromatographic methods of assay, were purchased from the California Foundation for Biochemical Research. Ammonium pyridoxal phosphate, obtained earlier from the same source, underwent rapid decomposition during storage and failed to prevent the inactivation of kynurenine transaminase (2).

Conditions of incubation and assay were similar to those described earlier (2). The incubation periods were 1 hour or less as indicated, the total volume of incubation mixture was 1 ml. The concentration of kynurenine was 0.0035 M and of keto acids, 0.006 M, except when indicated otherwise.

* A report of this study was presented before the American Society of Biological Chemists, Atlantic City, April 18, 1956 (1).

¹ Warnell, J. L., and Berg, C. P., unpublished. We are indebted to Dr. J. L. Warnell for the use of an ozonizer and an improved modification of a method for preparing kynurenine sulfate (3).

Transamination was measured by determining kynurenic acid formation and is expressed as the change (Δ^{333}) of optical density at 333 m μ after correction for the absorption due to kynurenine

Enzyme Purification—The preparation of the kynurenine holotransaminase is handicapped, since the enzyme is readily inactivated in slightly acidic buffer solutions (2). In the present study, it was found to be completely inactivated by ammonium sulfate fractionation (see below) and almost completely inactivated by alcohol fractionation and by freezing followed by thawing. Activity was restored in each case by the addition of small amounts of pyridoxal phosphate, indicating that inactivation was caused by dissociation of the coenzyme

The kynurenine apotransaminase, on the other hand, was fairly stable. Some purification of the apotransaminase was obtained as follows: 18.5 gm of rat kidney were homogenized with 75 ml of ice-cold water. The homogenate was centrifuged briefly to remove cell debris, and ammonium sulfate was added slowly to the supernatant fluid to bring it to 25 per cent of saturation. The precipitate was removed by centrifugation, and ammonium sulfate was added to the supernatant fluid to 75 per cent of saturation. The new precipitate was removed by centrifugation, dissolved in 30 ml of water, and dialyzed against 0.001 M phosphate buffer, pH 7.0, for 20 hours. More protein precipitated upon freezing followed by thawing, this was removed by centrifuging. The supernatant fluid had approximately 6 times as much apotransaminase activity per mg of dry weight as the original homogenate. It was inactive unless pyridoxal phosphate or pyridoxamine phosphate was added. The preparation was stored in the frozen state for 2 months without significant loss of kynurenine apotransaminase activity.

Results

As reported before (2), the kynurenine transaminase of rat kidney extracts became inactive within a few minutes at 37° in phosphate buffer, pH 6.3, unless α -ketoglutarate was present. At pH 7.4, on the other hand, inactivation was slow (Fig. 1). The rate of inactivation also depended upon the concentration of the phosphate buffer (Fig. 2). Subsequent addition of pyridoxal phosphate or pyridoxamine phosphate to the inactivated preparations restored the activity to levels which were higher than those that existed before preincubation.² Purified preparations which were completely inactivated during ammonium sulfate fractionation were similarly activated as illustrated in Fig. 3. When such reconstituted enzyme preparations were dialyzed in 0.002 M phosphate buffer, pH 7.4, to remove the unbound coenzyme, much of the activity was retained (Table I). These

² Preincubation is used here to designate a period of preliminary incubation during which the absence of one or more factors prevents transamination.

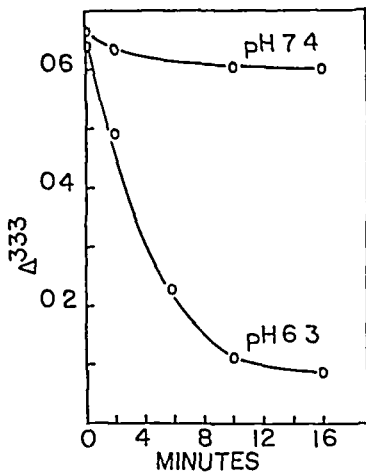


FIG 1

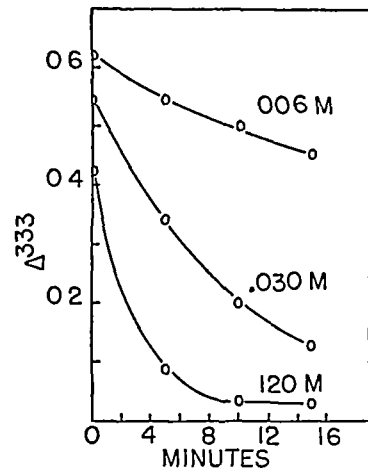


FIG 2

FIG 1 Effect of pH on the rate of inactivation of kynurenine transaminase during preincubation in 0.05 M phosphate buffer. The kidney extract was added to buffer solutions at zero time. α -Ketoglutarate was added after the intervals indicated by the points on the curves. After 16 minutes of preincubation, kynurenine was added to start the reaction.

FIG 2 Effect of phosphate buffer concentration on the rate of inactivation at pH 6.3. The experimental conditions were similar to those described in Fig 1 except for the variation of concentration of the phosphate buffer. pH measurements before and after incubation showed that the lower concentrations of buffer were adequate to prevent significant pH changes.

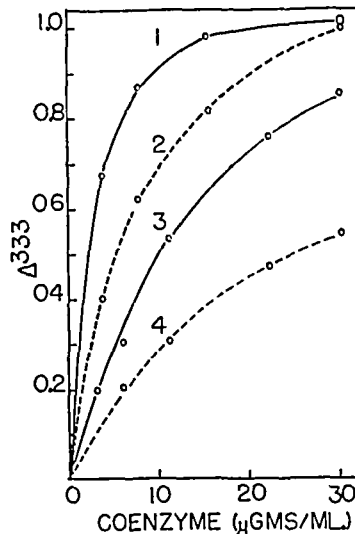


FIG 3 Relationship of activity to coenzyme concentration. Apoenzyme preparations were obtained by ammonium sulfate fractionation (see under "Materials and methods"). No preincubation. Solid lines, activation with pyridoxal phosphate, broken lines, activation with pyridoxamine phosphate. Curves 1 and 2, pH 6.3, Curves 3 and 4, pH 7.4.

dialyzed preparations, like the freshly prepared extracts, quickly lost their activity during preincubation in 0.1 M phosphate buffer, pH 6.3, unless α -ketoglutarate was present

These observations strongly indicate that the inactivation resulted from dissociation of the holoenzyme and that the dissociation was slowed or prevented (although not measurably reversed) by α -ketoglutarate. The protective action of α -ketoglutarate may be a result of a greater affinity of the apoenzyme for pyridoxal phosphate than for pyridoxamine phosphate, as shown by the higher concentrations of the latter needed to saturate the apoenzyme (Fig. 3). The scheme presented in Fig. 4 shows how the holoenzyme may be rendered unstable in the absence of keto acid. The portion

TABLE I
*Effect of Dialysis on Activity of Reconstituted Kynurenine Transaminase**

pH	Assay conditions, preincubation in 0.1 M buffer	Δ^{233}		
		Before dialysis	Dialyzed 11 hrs	Dialyzed 28 hrs
6.3	With α -ketoglutarate	0.441	0.299	0.273
	Without α -ketoglutarate	0.349	0.096	0.042
7.4	With α -ketoglutarate	0.370	0.232	0.190
	Without α -ketoglutarate	0.343	0.226	0.183

* Purified preparations obtained by ammonium sulfate fractionation (see under "Materials and methods") were incubated in 0.02 M phosphate buffer, pH 7.4, with pyridoxamine phosphate (45 γ per ml) for 2 hours at 37°, then dialyzed for 28 hours in the cold room in 400 volumes of 0.002 M phosphate buffer, pH 7.4. Kynurenine holotransaminase activity was assayed under the conditions and at the times indicated.

of Fig. 4 lying above the dotted line is believed to represent the situation before kynurenine and α -ketoglutarate are added. Amino acids present in the enzyme extract will tend to maintain the holoenzyme in the aminated form resulting in its rapid dissociation. Added α -ketoglutarate should retard dissociation by converting the holoenzyme to the more stable aldehyde form.³

Consistent with the above interpretation, the abilities of the various keto acids in preventing inactivation (Fig. 5) paralleled their effectiveness in transaminating with kynurenine (Table II). α -Ketoglutarate and oxalacetate were considerably more effective in both the protective and sub-

³ To simplify illustration and discussion, a two-step transamination mechanism (4) is assumed. The suggestion that α -ketoglutarate prevents inactivation by converting the holoenzyme to a form which does not dissociate readily may be valid even if the two step mechanism does not apply.

TABLE II
Comparison of Various α -Keto Acids As Substrates for
Kynurenine Transaminase Reaction*

Experiment No	Keto acid	Δ^{333}
1	α -Ketoglutarate	0 589
	Oxalacetate	0 528
2	α -Ketoglutarate	0 978
	α -Ketoisovalerate	0 038
	α -Ketoisocaproate	0 292
	α -Keto- β -methylvalerate	0 063
	Pyruvate	0 103
3	α -Ketoglutarate	0 900
	α -Ketovalerate	0 318
	α -Ketobutyrate	0 120

* Aliquots of a purified preparation obtained by ammonium sulfate fractionation (see under "Materials and methods") were incubated with 30 γ of pyridoxal phosphate and standard concentrations of keto acids and kynurenine sulfate in M/15 phosphate buffer, pH 6.3, for 1 hour

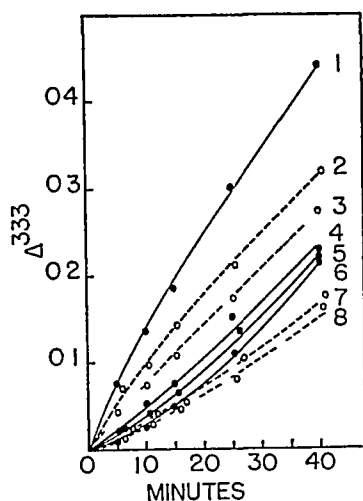


FIG 6

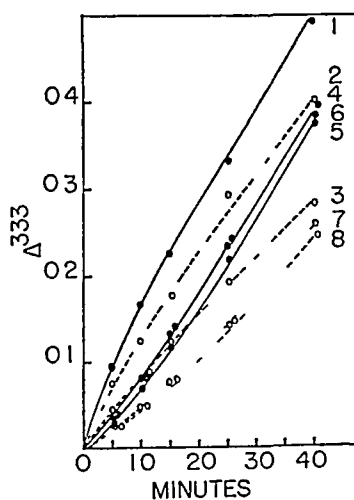


FIG 7

FIGS 6 AND 7 Effect of certain conditions during preincubation on the course of the subsequent kynurenine transaminase reaction. Fig 6, activation with pyridoxamine phosphate, 7.5 γ per ml. Fig 7, activation with pyridoxal phosphate, 7.5 γ per ml. Solid lines, pH 6.3, broken lines, pH 7.4. Preincubation condition, Curves 1 and 2, coenzyme plus α -ketoglutarate, Curves 3 and 4, coenzyme only, Curves 5 and 7, α -ketoglutarate only, Curves 6 and 8, neither. The curves for incubation mixtures which were not preincubated were like Curves 6 and 8 and are not shown. In all instances the reaction was started after 45 minutes by the addition of kynurenine and any other required factor not added earlier. Aliquots were removed and deproteinized after intervals indicated by the points on the curves.

activation At pH 7.4, on the other hand, such preincubation resulted in substantial increases (Curve 3), although these were not as great as when α -ketoglutarate was present Accordingly, there is both a lesser tendency to dissociate and a greater tendency to associate at pH 7.4 than at pH 6.3

Although the kynurenine transaminase of rat kidney appears to dissociate more readily than most enzymes involving pyridoxal phosphate, it may be paralleled in this respect by a system reported by Blakley (5) which interconverts serine and glycine This system became inactive at pH values above 9 and was reactivated by the addition of pyridoxal phosphate Inactivation was thought to involve adsorption of the coenzyme by inert constituents of the enzyme preparation Such an explanation has not been excluded in the present study, but it is believed to be of minor or secondary importance because little loss of kynurenine transaminase activity was evident after 45 minute preincubations of added coenzyme with apoenzyme preparations (Fig. 6) Cleavage of pyridoxal phosphate and pyridoxamine phosphate by a phosphatase in the kidney homogenates was demonstrated, however, by means of paper chromatography⁴ This cleavage was discounted as a major factor in the inactivation phenomenon under study because increases in the concentration of phosphate buffer in the reaction mixtures resulted in decreases in the rate of the phosphatase reaction, whereas they increased the rate of inactivation of the kynurenine holotransaminase in kidney extracts at pH 6.3 (Fig. 2)

DISCUSSION

The transamination system under study is like the glutamate-aspartate transaminase system (6) in that an appreciable time interval is needed for maximal activation by pyridoxamine phosphate at pH 7.4 and that dialysis at pH 7.4 does not readily resolve the reconstituted holotransaminase The kynurenine transaminase system, however, was maximally activated by pyridoxamine phosphate only after preincubation with α -ketoglutarate Such an effect of keto acids in the activation of a transaminase seems not to have been reported previously, although superficially similar effects have been noted in the activation of bacterial tryptophanase (7) and in decarboxylase (8) systems Judging from the nature of the reactions involved, pyridoxamine phosphate is probably not a coenzyme in the decarboxylase and tryptophanase systems, hence the necessity for its conversion to pyridoxal phosphate prior to activation The activating effect of keto acids in the kynurenine transaminase system, on the other hand, seems to be a result of the conversion of the apoenzyme-pyridoxamine phosphate complex to a more stable apoenzyme-pyridoxal phosphate complex Possibly the greater stability of the latter form arises from the anionic character of

⁴ Mason, M., unpublished data

pyridoxal phosphate at pH 6.3 (by calculating from dissociation constants obtained by Williams and Neilands (9)) contrasted with the isoelectric character predominating for pyridoxamine phosphate at that pH. The more strongly anionic character of both coenzymes at higher pH reactions may similarly explain the greater stability of the holoenzyme at pH 7.4.

SUMMARY

The previously described inactivation of the kynurenine transaminase of rat kidney which occurs when the extracts are incubated with phosphate buffer, pH 6.3, was shown to be strongly dependent on the concentration of phosphate buffer and to be completely reversed by the addition of small amounts of pyridoxal phosphate or pyridoxamine phosphate.

Pyridoxamine phosphate was much less effective than pyridoxal phosphate in activating the kynurenine apotransaminase. Inactivation was therefore interpreted to be a result of rapid dissociation of the pyridoxamine phosphate from the apoenzyme. Both α -ketoglutarate and oxalacetate served adequately as substrates at concentrations of 0.006 M and prevented inactivation at concentrations as low as 0.002 M. Various monocarboxylic α -keto acids, at similar concentrations, were relatively ineffective both in the protective and substrate functions. It was concluded that the keto acids prevent dissociation during incubation at pH 6.3 by maintaining the holoenzyme in the more stable apoenzyme-pyridoxal phosphate form.

The author wishes to express his appreciation to Mrs. Eva McKenna for assistance in purifying the apoenzyme preparations and to Mr. Clare Johnston for preparing kynurenine sulfate.

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THE METABOLISM OF THE ORGANIC ACIDS OF TOBACCO LEAVES

XIII EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF POTASSIUM BICARBONATE

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When organic acids which can readily enter into the metabolism of the leaves of the tobacco plant are supplied through the vascular system by the excised leaf culture technique, a marked accumulation of citric acid is frequently observed. This has been shown to occur after the administration of L-malate (1), succinate (2), fumarate (3), glycolate (4), and *d*-isocitrate (5). No evidence is seen for a stimulation of the formation of citric acid above the rate observed in control leaves when oxalate (6, 7) or (+)-tartrate (8) is furnished, and some interference with the reactions seems to be exerted by maleate (3), malonate (9), or acetate (5). The general effects are those to be anticipated if a system of metabolic reactions which has analogies with the tricarboxylic acid cycle is assumed to play an important role in the leaves of this species. The recent demonstration (9) of the rapid accumulation of succinic acid when malonate is administered to tobacco leaves indicates that an active succinic acid dehydrogenase system is present, an observation which furnishes further evidence in support of this view.

Nevertheless, many other systems of enzymatic reactions in which organic acids are involved also share in the general metabolism of plant leaves. Of the numerous possibilities open to investigation by the excised leaf technique, a study of the effect of the administration of bicarbonate ion in darkness was of particular interest, since the mechanisms concerned with the so called dark fixation of carbon would presumably become involved. The outcome has been the observation that citric acid accumulates in tobacco leaves cultured in 0.2 M potassium bicarbonate for 48 hours to about the same level as it does in identical leaves cultured in succinate or fumarate under similar conditions.

EXPERIMENTAL

Nicotiana tabacum, variety Connecticut 49, was grown in the greenhouse on soil with occasional additions of nutrient solution. The samples of

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leaves were collected on July 11, 1955, by the statistical method (10) 55 days after the seedlings were set out, ten samples of twenty leaves each being cut from twenty plants, the ten leaves counting upwards from the lowest full-sized leaf being taken from each plant. The coefficient of variation of the fresh weight was 2.5 per cent and that of the total nitrogen content was 1.14 per cent, the sampling error was thus acceptably small.

Of the four experimental samples, two were cultured for 24 hours with the bases of the leaves, respectively, in 0.1 M and in 0.2 M potassium bicarbonate, and two for 48 hours in the same solutions. Separate control samples were cultured for 48 hours in water, in 0.2 M potassium sulfate, in 0.2 M dipotassium phosphate at pH 8.0, in 0.2 M potassium succinate initially adjusted approximately to pH 7.0, and in 0.2 M potassium fumarate initially adjusted approximately to pH 6.9. The controls of the air-conditioned dark room were set for 24° and 50 per cent relative humidity. During the culture period, the reaction of the phosphate solution changed to pH 7.8, and readings on the succinate and fumarate solutions at 24 and 48 hours showed minor changes since these solutions were also inadequately buffered. It was assumed for purposes of calculation that the reaction of the succinate solution during most of the culture period was at pH 6.8 and that of the fumarate solution at pH 6.9. Readings on the bicarbonate solutions indicated that they were approximately at pH 9.

The control cultures in potassium sulfate and in water served to give information on the behavior of this set of samples under conditions that have been repeatedly examined, that in phosphate was designed to show the effect of a moderately alkaline salt solution, and the cultures in succinate and fumarate were intended to show the effect of almost completely neutralized solutions of these acids at pH reactions more alkaline than have previously been studied. The analytical methods employed have been described in previous papers (11, 12).

The changes in the fresh weight of the samples during culture are given in Table I, Line 1, all were negligibly small save for the leaves cultured for 48 hours in 0.2 M potassium phosphate at pH 8 and in 0.2 M potassium bicarbonate. The leaves in both of these samples wilted and lost about one-fifth of their initial weight, but there was no other obvious evidence of harm to the tissue. The increases in the ash in Line 2 show that only a moderate uptake of potassium bicarbonate occurred in comparison with the uptake of salts from the control solutions, and the increases in the alkalinity of the ash in Line 5 conform with this conclusion, as do the data for the increases in potassium¹ in Line 6. It may be noted that the increase in the alkalinity of the ash in the sample cultured in phosphate at pH 8

¹ We are indebted to the Department of Analytical Chemistry for the determinations of potassium by the flame photometric method.

should be one-half of the increase in potassium if dipotassium phosphate is converted into pyrophosphate in the muffle furnace, this was approximately true. However, the agreement between the data for the increases in the alkalinity of the ash and of the potassium is only moderately good, the mean discrepancy in the seven experiments being slightly more than 10 per cent of the amount of potassium found.

The effects upon the pH of extracts of the dried tissue (Line 7) were all small save for the samples cultured in phosphate and in 0.2 M bicarbonate for 48 hours, these became nearly 1 pH unit more alkaline.

The uptake of acids from the culture solutions (Line 8) was computed from the change in the alkalinity of the ash (Line 5) except for the sample cultured in phosphate. This was calculated from the increase in the ash on the assumption that the acquired dipotassium phosphate was converted into potassium pyrophosphate. The uptake of carbonic acid, expressed in milliequivalents, was taken to be twice the increase in the alkalinity of the ash, while that of succinic acid was calculated on the assumption that at pH 6.8 this acid is 97 per cent neutralized. At pH 6.9, fumaric acid was assumed to be completely neutralized.

Lines 10 to 17 (Table I) show the effects of the culture solutions upon the organic acid composition of the samples. Line 10 gives the data for the changes in the amounts of titratable organic acids that are eluted from the Dowex 1 column during the analytical procedure. The data for the changes of the separately determined oxalic acid (Line 13) are not included. The small losses from the samples cultured in water or in potassium sulfate presumably represent effects of respiration. The large increase in the sample cultured in phosphate represents the acquisition of a component which has been designated "Unknown acid A" in previous papers of this series. This component is eluted from the Dowex 1 column together with citric acid, and the quantity shown (Line 16) is in each case the difference between the sum of the titrations of the fractions that contain citric acid and the result of the determination of citric acid by the pentabromoacetone method (13) in the pooled fractions. The identification of this component as phosphoric acid was made by conventional qualitative and quantitative tests.²

Malic acid decreased and citric acid increased in the samples cultured in water and in potassium sulfate in the manner to be expected from previous experiments, and the amount of citric acid formed in the water control indicates that the enzyme systems concerned in these reactions were moderately vigorous. The loss of malic acid from the two samples cultured for 48 hours in bicarbonate was of about the same magnitude as the

² England and Colowick (14) have also recently observed that phosphoric acid is eluted from Dowex 1 by formic acid.

TABLE I

Effect upon Composition of Excised Tobacco Leaves of Culture in Solutions of Bicarbonate

The data represent gm or milliequivalents per kilo of initial fresh weight of tissue

Line No		Control before culture	Changes during culture in darkness												
			Water	K ₂ SO ₄	K ₂ HPO ₄	Potassium bicarbonate				K succinate	K fumarate				
						0.2 M	48 hrs	0.1 M	24 hrs			0.1 M	24 hrs	0.2 M	48 hrs
1	Change in fresh weight, %	0	+5.8	-0.4	-20.5	+3.0	+3.1	-1.9	-21.4			-1.8	+2.7		
2	Ash, gm	18.4	-0.5	+8.2	+13.5	+2.0	+2.2	+3.3	+5.0			+12.3	+9.5		
3	Organic solids, gm	71.9	-2.9	-3.8	-2.3	-2.6	-3.9	-2.9	-4.2			+1.7	-0.9		
4	“ “ corrected for CO ₂ of ash, gm	78.5	-3.1	-4.0	(-3.0)*	-2.0	-3.2	-1.8	-2.8			+5.4	+2.0		
5	Alkalinity of ash, m eq	299	-7.6	-9.5	+7.4	+28	+32	+49	+67			+170	+131		
6	Potassium, m eq	181	-11	+102	+164	+32	+39	+54	+74			+194	+136		
7	pH of extract of dry tissue	5.2	+0.1	+0.1	+0.8	+0.2	+0.2	+0.4	+0.9			+0.4	+0.2		
8	Uptake of acid, m eq				244	56	65	97	133			175	131		
9	“ “ “ gm					1.7	2.0	3.0	4.1			10.3	7.6		
10	Total titratable acids (exclusive of oxalic acid), m eq	194	-8	-14	+112	+21	+16	+25	+31			+154	+129		
11	Malic acid, m eq	150	-32	-35	-22	-11	-28	-13	-28			+55	-5		
12	Citric “ “	20.3	+20.1	+17.2	+30.5	+27.3	+39.2	+31.7	+58.0			+48.8	+54.3		
13	Oxalic “ “	24.1	+1.0	-0.6	+0.7	+0.9	+1.4	+0.8	+2.8			+4.4	+2.6		
14	Succinic acid, m eq	Trace										+41			
15	Fumaric “ “														
16	“Unknown acid A” (H ₃ PO ₄), m eq	15.3	+2.0	+3	+103	+2	+3	+1	-1			+1	+74.5		
17	Phosphoric acid, † m eq	23.9			+179								-2		
18	Undetermined acids, m eq	8.8	+1.0	+0.4	+0.4	+2.6	+2.3	+4.6	+2.2			+7.7	+6.2		
19	Protein N, gm	2.46	-0.20	-0.38	-0.29	-0.13	-0.23	-0.15	-0.38			-0.34	-0.38		

* The correction in this instance is for the conversion of K₂HPO₄ to K₄P₂O₇ during the combustion of the sample used for the determination of the ash † Phosphoric acid in the pooled citric acid fractions

loss from the water control, but the increases of citric acid were notably greater. Malic acid increased in the sample cultured in succinate at pH 6.8 as it has been found to do both at pH 5 (3) and pH 6 (7), and the formation of citric acid was also stimulated. The effect of culture in fumarate at pH 6.9 upon both malic acid and citric acid also closely resembled the effects previously observed at pH 5 and 6 (3).

The oxalic acid content of the samples (Line 13) was changed little if at all by any of the conditions of culture. The presence of a trace of succinic acid could be detected in most of the samples on the plots of the titration of the fractions eluted from the Dowex 1 column. However, only the sample cultured in succinate contained a significant amount of this acid (Line 14). No fumaric acid was detected in any of the samples except the last, the fractions eluted immediately after citric acid during the analysis of this sample each left a visible deposit of fumaric acid when dried in preparation for titration (Line 15).

The component hitherto designated Unknown acid A (Line 16) found in the pooled citric acid fractions from the Dowex 1 column and now identified as phosphoric acid³ was not influenced by any of the conditions of culture with the exception of the sample cultured in phosphate. A water extract of the control sample contained the equivalent of 27.1 m eq of phosphoric acid per kilo by the Fiske and Subbarow method (15), and the pooled citric acid fractions obtained from it contained 23.9 m eq. Thus, 88 per cent of the phosphoric acid initially present found its way into the citric acid fraction. At the end point of the indicator used in the titration of these fractions, phosphoric acid is about 66 per cent titrated. Accordingly, 15.9 m eq of acidity due to phosphoric acid should have been titrated, the quantity of Unknown acid A found was 15.3 m eq. In the citric acid fraction from the sample cultured in phosphate, the increase in phosphoric acid was 17.9 m eq. The increase in acidity should therefore have been 11.9 m eq, the increase found being 10.3 m eq.

The item designated "Undetermined acids" in Line 18 represents the sum of the titrations of the fractions eluted by formic acid from the Dowex 1 column in advance of malic acid. This is a complex mixture which contains trace amounts of D-glyceric acid (16), succinic acid, quinic acid, aspartic acid, glycolic acid, and several other components as yet unidentified. Succinic acid can usually be recognized since it is the last to emerge before malic acid. For the detailed examination of this group of acids, the use of acetic acid as eluting agent is essential (12). The data in Line 18 suggest that bicarbonate ion supplied to the leaves exerts a little in-

³ In experiments on a larger scale, traces of malonic and of *d*-isocitric acids have also been detected in this fraction by chromatographic and enzymatic methods.

fluence upon these substances, succinic and fumaric acids, however, give rise to a marked increase in one or more of them

TABLE II

Metabolic Effects of Bicarbonate Ion on Composition of Tobacco Leaves

Data expressed in terms of 1 kilo of initial fresh weight of leaves For an explanation of the derivation of these quantities, see the text

Line No		Changes during culture in darkness								
		Water	K ₂ SO ₄	K ₂ HPO ₄	Potassium bicarbonate				K suc- cinate	K fu- marate
			0.2 M	0.2 M pH 8.0	0.1 M	0.1 M	0.2 M	0.2 M	0.2 M pH 6.8	0.2 M pH 6.9
			48 hrs	48 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	48 hrs
1	Respiration loss, gm	3.1	4.0	(3.0)	(3.7)	(5.2)	(4.8)	(6.9)	4.9	5.6
2	Δ ash found, gm	-0.5	+8.2	+13.5	+2.0	+2.2	+3.3	+5.0	+12.3	+9.5
3	Δ potassium cal- culated as salt (K ₂ SO ₄ , K ₄ P ₂ O ₇ or K ₂ CO ₃), gm		+8.9	+13.6	+2.2	+2.7	+3.7	+5.1	+13.4	+9.4
4	K ₂ CO ₃ equivalent to Δ alkalinity of ash, gm				1.9	2.2	3.4	4.6	11.8	9.0
5	Δ malic Δ citric acids, molar ra- tio	2.4	3.0	1.1	0.6	1.1	0.6	0.7		0.2
6	Citric acid as- sumed to be de- rived from malic acid, mmoles	7.9	8.7	5.5	2.9	7.1	3.2	6.9		1.9
7	Citric acid de- rived from another source, mmoles	-1.2	-3	4.7	6.2	6.0	7.3	12.4		16.2
8	Uptake of acid, mmoles				28.0	32.5	48.5	66.5	87.5	65.5
9	Ratio, Line 7 to Line 8, as %				22.2	18.4	15.6	18.6		0.25
10	Ratio, Δ titratable acids to twice data of Line 8, as %				36.8	24.5	25.2	23.6		

The data for the behavior of the protein (Line 19) indicate that proteolytic reactions were stimulated in the leaves cultured in solutions of salts as has been repeatedly observed. No effect attributable to the alka-

limity of the solutions used in the present experiments was noted. The data for the behavior of the starch content of the leaves are not given in detail. The leaves contained only 0.70 gm per kilo of starch at the start of the experiments, an unusually small amount, and only traces remained at the end.

In Table II are collected data derived from the analytical results of Table I. The estimations of respiration loss (Line 1) represent in each case the difference between the change in the corrected organic solids and the calculated uptake of acid. The loss from the water control sample was rather small. However, the administration of potassium sulfate or of the salts of the organic acids resulted in an increase in the net loss of organic substances from the leaves as is usually observed. The loss from the sample cultured in phosphate was computed from the weight of dipotassium hydrogen phosphate equivalent to that of the pyrophosphate in the ash. This was deducted from the total solids to give an estimate of the organic solids in this sample. No further correction for carbonate in the ash was made and the value found for the respiration loss is accordingly parenthesized. If such a correction is applied, the apparent loss becomes 1.4 gm per kilo, an improbably low result. The estimates of respiration loss from the samples cultured in bicarbonate are also parenthesized since they were made on the assumption that the bicarbonate ion taken up from the alkaline culture solution was retained in the tissue, *i.e.*, that no escape of carbon dioxide occurred from a medium initially at or near pH 5.2. This seems unlikely and, accordingly, the values given merely furnish an upper limit for the respiration loss. The uncertainty results from the unsuitability of the experimental technique for studies of the effects of the administration of a volatile acid when precise values for the quantities fixed by the enzyme systems are required.

Lines 2 to 4 of Table II show the increases in the inorganic solids, calculated from the determinations of potassium and of alkalinity of ash, in comparison with the increases of ash found. The agreement is satisfactory in most instances.

The molar ratio of the decrease in malic acid (Table I, Line 11) to the increase in citric acid (Table I, Line 12) is given in Line 5 of Table II. The value 2.4 for the sample cultured in water is within the limits usually found and indicates that the metabolism of these acids followed the normal course in this set of samples. The ratio 3.0 for the sample cultured in potassium sulfate is a little high although not greatly out of line. It is possible that the utilization of malic acid was somewhat stimulated. The ratios for the other samples are, however, all seriously depressed and clearly reveal a marked interference with the usual course of events. No explanation can at present be advanced for the pronounced effect of phosphate

Lines 11 and 12 of Table I show that the utilization of malic acid by this sample, in comparison with the control, was somewhat low, while the formation of citric acid was stimulated. The effects upon the organic acids of tobacco leaves of culture in solutions of phosphate obviously warrant further study.

The advent of bicarbonate ion did not diminish the extent to which malic acid was utilized to any important degree, but there was a marked stimulation of the formation of citric acid. Twice as much citric acid was present in the leaves cultured for 48 hours in 0.1 M bicarbonate as was found in the water control, and about three times as much in those cultured in 0.2 M bicarbonate. Inquiry into the details of this effect is rendered difficult because of the lack of data on the amounts of bicarbonate ion which actually entered into the reactions of the cells. If it is assumed, however, that the malic acid which disappeared from these samples was converted into citric acid in the same ratio as it is in leaves that have been cultured in water, some information regarding this quantity can be obtained. Line 6 of Table II shows the amounts of citric acid that would have resulted if 2 moles of malic acid were consumed in such a sequence of reactions, and Line 7 the estimated amounts of citric acid that must therefore have had some other origin. The corresponding uptakes of carbonic acid are shown in Line 8. Line 9 then gives as percentages the molar ratios between these respective amounts of carbonic acid and the amounts of citric acid assumed to have resulted from reactions in the course of which the acquired carbon dioxide entered into the formation of carboxyl groups.

If a single carboxylation reaction was involved in the synthesis of each mole of citric acid produced, molar quantities of carbonic acid equal to those of citric acid shown in Line 7 would be required. The data in Line 9 thus imply that, as the mean of the four observations, 18.7 per cent of the carbonic acid taken up was converted into non-volatile acids, the remainder presumably being lost as carbon dioxide. If this figure is used in the calculation of the respiration loss, the estimates for these four samples in Line 1 of Table II are diminished, respectively, to 2.3, 3.5, 2.4, and 3.5. In view of the small uptake of bicarbonate, these are entirely reasonable magnitudes. If more than one carboxylation reaction is involved in the synthesis of citric acid, or if the efficiency with which the bicarbonate ion was used was greater than about 19 per cent, these figures become somewhat larger.

DISCUSSION

The observation that the administration of bicarbonate ion to the leaves of the tobacco plant promotes the formation of citric acid to about the same extent as succinate or fumarate under the same conditions is sug-

gestive of the operation of a mechanism whereby one or the other of these substances or, more probably, a closely related acid is produced by means of a carboxylation reaction. The product may then be supposed to enter into the sequence of metabolic reactions characteristic of this species, the ultimate accumulation of citric acid being a consequence of the fact that citric acid is utilized at a rate considerably slower than it is formed. Granted a source of pyruvic acid from the metabolism of the carbohydrates, of which an adequate supply was available, the starch alone being sufficient in all except one instance, the widely distributed (17) malic enzyme of Ochoa, Mehler, and Kornberg (18) provides a mechanism whereby malic acid might arise from bicarbonate. A mechanism such as the formation of oxalacetic acid from phosphoenolpyruvic acid and carbon dioxide, observed by Bandurski and Greiner (19) in spinach leaves and studied by Tchen and Vennesland (20), is an additional possibility, and other suggestions can also be made. However, oxalacetic acid has not been detected in the tobacco leaf and if formed would presumably have only a transitory existence, since enzymes such as malic dehydrogenase (21) which rapidly transform it to other substances are known to be present. Whatever the precise mechanism may be, Stutz and Burris (22) have demonstrated that radioactive carbon dioxide can be acquired by young tobacco plants from the surrounding air and assimilated into malic acid in darkness, and the present experiments provide direct analytical evidence of the operation of a mechanism whereby carbon dioxide is fixed as carboxyl groups.

If the mechanism by which citric acid is formed as a result of the metabolism of bicarbonate ion is in fact one which involves an interaction with pyruvic acid derived from the metabolism of the carbohydrates, a second method to estimate the proportion of the acquired bicarbonate ion which entered into the reactions becomes possible. The increase in the titratable organic acidity in Table I, Line 10, furnishes a measure of the new carboxyl groups and, if the assumption is valid, one-half of these were derived from bicarbonate ion and the other half from pyruvic acid. Accordingly, the ratio of the increase in titratable acidity to twice the uptake of bicarbonate ion (Table II, Line 8) yields the fraction of the available bicarbonate ion which was fixed as carboxyl groups. This ratio, expressed as a percentage, is given in Table II, Line 10. Of the four values, three agree with each other closely and suggest that approximately 24 per cent of the bicarbonate ion acquired by the tissues entered into the metabolic reactions. The agreement with the estimate of about 19 per cent derived from other data upon quite different assumptions is perhaps as close as could be expected.

The presence of one further enzyme mechanism must be assumed in

order to account for the formation of citric acid by the metabolism of one or other of the 4-carbon dicarboxylic acids, namely a system which has the same function as the condensing enzyme of Ochoa, Stern, and Schneider (23). Few attempts to demonstrate the presence of this specific enzyme in green leaves have been made and none has led to entirely convincing results, although Brummond and Burris (24) have obtained strongly suggestive evidence of its presence in lupine leaves. Nevertheless, the direct analytical evidence that citric acid is formed in the tobacco leaf at the expense of 4-carbon dicarboxylic acids is now extremely strong and is confirmed by the experiments of Zbinovsky and Burris (25) with radioactive malic acid.

Data which support the view that the general plan of the metabolic transformations of bicarbonate, succinate, fumarate, and L-malate in the tobacco leaf is essentially the same are collected in Table III. A third significant figure is carried as an aid in the calculations. The last column gives details from an earlier experiment (1) in which potassium L-malate was administered to tobacco leaves at pH 7. Lines 1 and 2 give the changes in citric and malic acids after 48 hours of culture in darkness and Line 3 the corresponding uptakes of acid from the respective culture solutions. Line 4 shows, for the experiments with bicarbonate, the two estimates of the amounts of bicarbonate ion metabolized if either 18.7 or 24 per cent of the amount taken up was fixed. The data for the other three experiments represent the differences between the amounts of the individual acids taken up and those found in the leaves after 48 hours. In Line 5, the assumption is made that in each case malic acid is actually or potentially the source of the citric acid formed. Thus, to the quantities of carbonic acid metabolized in the first two experiments are added the quantities of malic acid that were used up during 48 hours. The increase of malic acid in the experiment with succinate is deducted from the amount of succinic acid metabolized (Line 4) on the assumption that this amount of malic acid was derived from the acquired succinic acid and remained as such. The correction for the experiment with fumarate is small since only a little of the malic acid present at the start was drawn upon. In Line 6, a further correction is made on the assumption that the small over-all loss of titratable organic acids which occurred during the culture period fell exclusively upon the acquired acid. Line 7 then gives the ratios of the actual or potential malic acid which were presumably converted into citric acid to the increases in citric acid observed.

The constancy of these ratios is striking and it is of interest that the magnitude for succinate in the present experiment is identical with that found by a similar calculation in a number of previous experiments with this acid (3). The ratio for fumaric acid also agrees well with previous obser-

vations (3) The evidence thus points strongly to the conclusion that bicarbonate ion was metabolized in the tobacco leaf into a 4-carbon dicarboxylic acid, although whether this acid was malic acid or oxalacetic acid is impossible to determine from the present evidence

TABLE III

Consequences of Assumption That Bicarbonate Ion Furnished to Tobacco Leaves Is Assimilated into Malic Acid

Data expressed in terms of 1 kilo of initial fresh weight of leaves For an explanation of the derivation of these quantities, see the text

Line No		Changes during culture for 48 hrs in darkness				
		KHCO ₂	KHCO ₂	K succinate	K fumarate	K malate*
		0.1 M	0.2 M	0.2 M pH 6.8	0.2 M pH 6.9	0.2 M pH 7
1	Δ citric acid, mmoles	+13.0	+19.3	+16.3	+18.1	+13.8
2	Δ malic acid, mmoles	-14.1	-13.9	+27.7	-2.7	+36.9
3	Uptake of acid, mmoles	32.4	66.6	87.5	65.5	70.1
4	Acquired acid metabolized, mmoles	6.1 or 7.8†	12.4 or 16.0†	67.1	28.3	33.2
5	Acid metabolized corrected for Δ malic acid, mmoles	20.2 or 21.9†	26.3 or 29.9†	39.4	31.0	
6	Same corrected further for overall loss of acid, mmoles	20.2 or 21.9†	26.3 or 29.9†	28.7	30.1	23.9
7	Ratio, Line 6 to Line 1	1.55 or 1.68†	1.36 or 1.54†	1.76	1.66	1.73

* Data from an experiment with a different set of samples (1)

† Alternative values on the assumption that either 18.7 per cent or 24 per cent of the bicarbonate ion available entered into the metabolism (see Table II, Lines 9 and 10)

It is not maintained that the coincidence of these ratios demonstrates the validity of the assumptions involved in the calculations. Their significance at the present time lies mainly in what they reveal of the analogies in the general course of the metabolism of the respective substances in the tobacco leaf. The relationship between the quantity of citric acid formed

and that of its immediate precursor seems to be essentially constant. Difficulties arise, however, with the view that this amounts to a statement that the sequence of reactions is precisely that characteristic of the tricarboxylic acid cycle, and that succinic, fumaric, and malic acids are equivalent in their ultimate effect inasmuch as they are readily interconvertible substances. The third column of data in Table III shows that 67 mmoles of succinic acid were converted into other acids, 28 mmoles of malic acid and 16 mmoles of citric acid being formed. Accordingly, if the sequence of reactions is that of the cycle, all of this succinic acid at some stage must have been at least potentially present as fumaric acid. In the parallel experiment, 28 mmoles of fumaric acid were metabolized, no malic acid was formed, but 18 mmoles of citric acid accumulated. Furthermore, 37 mmoles of fumaric acid (Table I, Line 15) remained as such in the tissues although none was detected in the experiment with succinic acid. Obviously there are many details of the transformations which these acids undergo in the green leaf which are yet to be explained.

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SUMMARY

When excised leaves of the tobacco plant (*Nicotiana tabacum*, variety Connecticut 49) are cultured in darkness in potassium bicarbonate solution, from one-fifth to one-quarter of the bicarbonate ion taken up is fixed as the carboxyl groups of non-volatile organic acids. A stimulation of the formation of citric acid occurs which is approximately the same as that observed when succinate or fumarate is made available to the leaves from solutions at approximately pH 7. A consideration of the quantities of acquired acid that are metabolized and of the products of the reactions that occur leads to the conclusion that the molar relationship of the amount of precursor used to that of the citric acid formed is essentially constant whether bicarbonate, succinate, fumarate, or L-malate is made available to the enzyme systems of the leaves. Nevertheless, fumaric acid does not accumulate in the leaves when succinic acid is supplied although it does when fumaric acid is made available. Thus, although the general plan of the metabolic reactions resembles that of the tricarboxylic acid cycle, certain details remain to be explained.

It was observed incidentally that the administration of phosphate at pH 8 to the leaves stimulates the formation of citric acid. Furthermore,

the greater part of the inorganic phosphate of the leaves is retained by the Dowex 1 column used for the analytical determination of the organic acids and is eluted by formic acid together with citric acid

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STUDIES ON THE ANTIPROTEOLYTIC ACTIVITY OF BOVINE BLOOD

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It has been known for some time that mammalian blood inhibits trypsin and plasmin. The problem of the specificity of this antiproteolytic activity is still not resolved. Several investigators (1-5) have shown evidence of the presence in human blood of different inhibitory agents which vary in their specific effect on trypsin and on plasmin.

The present work is concerned with a comparison of the antitryptic and antiplasmin activities of various bovine plasma fractions, casein being used as a substrate. The ratio of antitryptic to antiplasmin activity for the various inhibitor fractions prepared was used as an index of whether all of the antiproteolytic activity of beef blood against trypsin and plasmin is due to one inhibitor or to separate proteolytic inhibitors. The data obtained are in agreement with the assumption that the antiproteolytic activity of beef blood against trypsin and plasmin is exerted by a single inhibitory factor.

EXPERIMENTAL

Preparation of Various Antiproteolytic Fractions from Bovine Plasma—The procedure for obtaining inhibitor Fractions I and II is similar to that employed by Loomis *et al* (6) and by Peanasky and Laskowski (7).

Inhibitor Fraction I—Oxalated bovine blood was collected at the slaughter house and centrifuged at 4°. To 1 liter of oxalated plasma, cooled to 0-5°, were added 1500 ml of 0.9 per cent sodium chloride. After thorough mixing, 605 gm of ammonium sulfate were slowly added with stirring and the solution was allowed to stand at 0-5° for at least 4 hours. After centrifugation in the cold for 45 minutes at 1300 × *g*, the supernatant fluid was adjusted to pH 3.7 to 3.8 with 5 *N* sulfuric acid, stirred for 10 minutes at 4°, and centrifuged at 4° for 45 minutes at 1300 × *g*. The precipitate was discarded and the supernatant fluid immediately adjusted to pH 6 to 7 with 5 *N* sodium hydroxide. To each 100 ml of solution, 28.05 gm of ammonium sulfate were slowly added with constant stirring. After standing for about 4 hours in the refrigerator, the solution was centrifuged. The supernatant fluid was discarded and the precipitate was dissolved in a

minimum of distilled water and dialyzed against distilled water at 4°. After dialysis, the protein concentration of the solution was adjusted to 1 per cent by the addition of distilled water, and 42 gm of ammonium sulfate stirred in, at room temperature, for each 100 ml of solution. After standing at room temperature overnight, the solution was centrifuged and 7 gm of ammonium sulfate for each 100 ml of the original 1 per cent protein solution were added to the supernatant fluid. After 4 to 6 hours at room temperature, the solution was centrifuged and the resulting precipitate dissolved in a minimal amount of distilled water. This solution was dialyzed against distilled water at 4° and lyophilized (inhibitor Fraction I).

Inhibitor Fraction II—Inhibitor Fraction I was dissolved in 0.9 per cent sodium chloride at a protein concentration of 1 per cent. For each 100 ml of the solution, 35 gm of ammonium sulfate were added with stirring at room temperature. Any precipitate formed was removed by centrifugation and the supernatant fluid, after being cooled to 0°, was adjusted to pH 4.0, stirred for 10 minutes at 0°, and centrifuged in the cold, and the supernatant fluid was adjusted to pH 7.0. 7 gm of ammonium sulfate were added for each 100 ml of the initial solution and the mixture was held at room temperature for 4 to 6 hours. Any resulting precipitate was removed by centrifugation and 14 gm of ammonium sulfate for each 100 ml of the initial solution were added. The entire procedure was repeated three times or until no precipitate was formed, except in the final fraction, which precipitated between 60 to 80 per cent saturation with ammonium sulfate. The final precipitate was dissolved in distilled water, dialyzed against distilled water at 4°, and lyophilized (inhibitor Fraction II).

Electrophoretic Separation of Inhibitor Fraction II—A continuous paper electrophoresis technique, as described by Selden and Westphal (8), was employed for further fractionation of inhibitor Fraction II. The preparation was dissolved in Michaelis buffer (pH 8.6, $\mu = 0.05$) at a protein concentration of about 6 per cent. The solution was fed on to Whatman 3 MM paper for 94 hours at an applied potential of 225 volts. The stained electrophoresis pattern showed essentially four fractions of different mobilities.

Heat Inactivation Studies—Inhibitor Fraction I or II, in concentrations of 1.2 per cent in tris(hydroxymethyl)aminomethane (Tris)-NaCl buffer,¹ was heated at various temperatures and lengths of time, as indicated in Table II, cooled rapidly in ice water, and tested. For antiproteolytic as-

¹ Tris-NaCl buffer of pH 7.25. 12.5 gm of Tris were dissolved in 500 ml of distilled water. After addition of 20 gm of sodium chloride and 85 ml of 1 N hydrochloric acid, the volume was brought to 2 liters and the pH, if necessary, was adjusted to 7.25.

says, the solutions were diluted with Tris-NaCl buffer to appropriate concentrations and the antiproteolytic activity of the heated material was compared with that of unheated control samples

Enzyme Preparations—Trypsin, a stock solution containing 3 to 4 mg of crystalline salt-free trypsin² in 100 ml of 0.0025 N hydrochloric acid, was used. Plasmin, 200 gm of dried human plasma, Fraction III,³ was finely ground and extracted with 4 liters of 0.2 N sulfuric acid for half an hour at room temperature (9). About 10 gm of Celite were added to the mixture, which was filtered rapidly through a Buchner funnel. The filtrate was immediately adjusted to pH 7 to 7.5 with 4 N sodium hydroxide, and ammonium sulfate was added slowly with stirring to 20 per cent of saturation (14 gm of salt for each 100 ml of solution). The solution was filtered as before, and the same amount of ammonium sulfate used in the first precipitation was added to the filtrate. The mixture was allowed to stand at 4° for 3 to 4 hours, and the precipitate, obtained after centrifugation at 4° for 1 hour at $1300 \times g$, was dissolved in 300 ml of 0.066 M phosphate buffer of pH 7.2. To the solution were added 3000 units of streptokinase⁴ for each gm of human plasma Fraction III used as starting material. After standing for 15 to 30 minutes at 25–26°, the mixture was adjusted to pH 3.0 with 1 N hydrochloric acid and dialyzed against 0.001 N hydrochloric acid at 4° until it was free from sulfate ions. The crude plasmin, obtained after lyophilization, was made up to a 1 per cent solution in 0.001 N hydrochloric acid. Sodium chloride was added to a concentration of 3 per cent and the mixture was kept at 4° for 3 to 4 hours. Any precipitate formed was removed by centrifugation and the supernatant fluid was brought to 20 per cent sodium chloride concentration. After standing for 2 hours at 4°, the precipitate was removed by centrifugation, dissolved in 0.001 N hydrochloric acid, dialyzed against 0.001 N hydrochloric acid in the cold, and lyophilized. This partially purified plasmin preparation was soluble in distilled water. It was found that the proteolytic activity of this material was of an order similar to the human plasmin preparation⁵ of Fishman and Kline (10), who employed a somewhat different purification procedure.

Casein Substrate—A 1 per cent casein (11) solution was prepared by heating 1 gm of casein in 100 ml of pH 7.4 phosphate-saline buffer at 100° in a water bath for 15 minutes. After filtration from traces of insoluble material, the solution was stored in a refrigerator.

² Obtained from Worthington Biochemical Corporation, Freehold, New Jersey

³ Obtained through the courtesy of the American National Red Cross and kindly supplied to us by the Cutter Laboratories

⁴ Streptokinase-streptodornase varidase was supplied to us through the generosity of Lederle Laboratories Division, American Cyanamid Company

⁵ Kindly supplied to us by Dr. Daniel L. Kline, Yale University School of Medicine, New Haven, Connecticut

Antiproteolytic Test—A modification of the methods of Northrup *et al* (12) and of Remmert and Cohen (13) was employed for determining proteolytic and antiproteolytic activities with casein as substrate

To 1 ml of the buffered inhibitor solution was added 1 ml of standardized plasmin or trypsin solution ⁶ Inhibitor and enzyme were permitted to react for 30 minutes at 25–26°, 2 ml of 1 per cent casein solution were then added and the mixture was incubated for 30 minutes at 35° ± 1° Proteolytic activity was stopped in the blanks containing enzyme and inhibitor immediately after the addition of the casein solution, and in the samples after 30 minutes incubation, by the addition of 5 ml of 5 per cent trichloroacetic acid The tubes were allowed to stand at room temperature for 2 hours with occasional shaking The precipitate was then removed by filtration through Schleicher and Schuell No 589 filter paper The optical density of the filtrates was read at 280 mμ against appropriate blanks in a Beckman DU spectrophotometer All antiproteolytic activity determinations were carried out in duplicate or triplicate

Definition of Proteolytic and Antiproteolytic Activities—10 trypsin or 10 plasmin units were arbitrarily defined as that degree of proteolytic activity which would produce an optical density of 0.500 after incubation with casein for 30 minutes at 35° ± 1° It was found that 68 γ of the crystalline trypsin used corresponded to 10 proteolytic trypsin units Correspondingly, 1 antitrypsin or 1 antiplasmin unit would be equal to that amount of the inhibitor preparation capable of inhibiting 1 unit of trypsin (6.8 γ) or of plasmin, respectively Curves obtained by plotting optical density against the enzyme units were used to calculate the antiproteolytic activity of an inhibitor preparation for trypsin and for plasmin, respectively The most reliable determinations were found to fall in the linear portion of the curves

In the assay, the difference between the number of proteolytic units in the standard enzyme solution and in the enzyme solution containing the inhibitor is indicative of the number of enzyme units inhibited The antiproteolytic activity of an inhibitor fraction has been expressed as antitrypsin and antiplasmin units per mg of preparation and is the basis for the calculation of the ratio of antitryptic to antiplasminic activity

Results

The average value of the antitrypsin to antiplasmin ratio for the various preparations reported, as well as others not included, was found to be 11 ± 2 As may be seen from Table I, the ratio between antitryptic and antiplasminic activity was 11.1 for beef serum Several investigators (14–16)

⁶ It was found that solutions containing approximately 30 γ of trypsin or 2 mg of plasmin per ml were most suitable for these antiproteolytic studies

have already reported that the inhibition of plasmin requires a higher concentration of serum or plasma than is necessary to inhibit trypsin of equal proteolytic potency

Should any separation of the antitryptic and antiplasmin activities present in beef blood occur during the various stages of purification or heat inactivation, one would expect a corresponding change in the antitrypsin to antiplasmin ratio as established for beef serum. Inhibitor Fractions I and II, obtained by ammonium sulfate fractionation of bovine plasma, gave

TABLE I
Antiproteolytic Activity of Bovine Serum and Various Plasma Fractions

Material	Antitrypsin units	Antiplasmin units	Antitrypsin- antiplasmin ratio
	<i>per mg protein</i>	<i>per mg protein</i>	
Beef serum*	310*	28.0*	11.1
Inhibitor Fraction I	13.3	1.4	9.9
“ “ II	31.0	2.4	12.8
Electrophoresis starting material	28.0	2.3	12.0
Electrophoretic Fraction A	5.5	0.5	11.0
“ “ B	12.0	1.0	12.0
“ “ C	18.0	1.6	12.0
“ “ D	74.0	5.6	13.0
“ “ E	79.0	7.2	11.0

The values given for serum, Fractions I and II, are the average of four and, for the electrophoretic fractions, of two experiments. Electrophoretic Fractions A, B, and C refer to the middle components collected and Fractions D and E are the fast moving components. The antiproteolytic activities of the slow as well as the fastest moving components were negligible and, therefore, are not recorded.

*Values for beef serum are given in units per ml

a ratio of 9.9 and 12.8, respectively, falling within the range of the ratio established for bovine serum (Table I)

Table I also presents the results for the fractions obtained by electrophoretic separation. Most of the antiproteolytic activity was obviously concentrated in the fast moving component. The antitryptic activity, found for Fractions D and E, would indicate that 1 mg of these inhibitor preparations would inactivate about 0.5 mg of crystalline trypsin. The data of Table I also illustrate the point that no significant change in the antitrypsin to antiplasmin ratio was found for any of the fractions obtained by electrophoresis.

Table II presents the results of experiments in heat inactivation with inhibitor Fractions I and II. The data show that the antitrypsin to antiplasmin ratios for the various heated preparations remained reasonably con-

stant, the variations being well within the range of experimental error. It would appear, therefore, that the thermal destruction of antitryptic and antiplasmin activities occurred at the same rate. It would seem that the more highly purified inhibitor Fraction II was somewhat more heat-stable than the inhibitor Fraction I (Table II).

Shulman (2) reported that heating of human serum at 60° for 20 minutes decreased antitryptic activity to 10 per cent of its original value, while the ability to inhibit plasmin remained practically unchanged. As may be seen from Table II, comparatively little loss in antitryptic activity of inhibitor Fractions I and II occurred when they were heated at 60° for 45

TABLE II
Heat Inactivation of Antiproteolytic Activity

	Material		Antitrypsin units	Antiplasmin units	Antitrypsin antiplasmin ratio
	°C	min	per mg protein	per mg protein	
Inhibitor Fraction I	Control unheated		14.3	1.5	9.6
	60	45	11.0	1.2	9.2
	65	45	8.3	0.7	11.7
	70	20	4.0	0.3	11.8
	70	30	2.5	0.2	10.3
Inhibitor Fraction II	Control unheated		31.0	2.4	12.7
	60	45	23.0	2.2	10.5
	65	45	28.0	2.2	12.7
	70	30	13.0	1.2	10.8

Values given are an average of four experiments

minutes. It is quite possible that human blood may contain a heat-labile specific antitryptic factor which, apparently, is not present in bovine blood. Recently Shulman (17) described an antitrypsin preparation from human plasma which is comparatively heat-stable. The possible identity of this inhibitor with the antiproteolytic factor from bovine blood has still to be elucidated.

DISCUSSION

The present findings are in agreement with the assumption that the antiproteolytic activity of bovine blood, measured against trypsin and plasmin, is due to a single inhibitor. In contrast with this finding is the evidence for the presence in human blood of different proteolytic inhibitors which differ in their specific effect on trypsin and plasmin (1-5). It is obvious from the data presented that, in order to inactivate a given amount of proteolytic plasmin activity, it was necessary to use approximately 10 times

as much inhibitor preparation as for an equal amount of trypsin activity measured on casein. It may, therefore, be possible to observe mainly antitryptic activity and comparatively little antiplasmin activity if the amounts of the inhibitor preparation employed in the antiplasmin assay are not within the proper concentration range. This ratio of antiplasmin to antitryptic activity would have to be taken into account when the antiplasmin activity of an inhibitor preparation is measured against human plasmin.

SUMMARY

The results of experiments designed to separate antitryptic and antiplasmin activities of bovine blood by ammonium sulfate fractionation, by electrophoresis, and by heat inactivation agree with the assumption that the antiproteolytic activity of bovine blood against trypsin and human plasmin is due to a single inhibitory factor.

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ON THE NATURE OF THE INHIBITION OF GLUTAMATE OXIDATION BY THE CARCINOGEN, *N*-2-FLUORENYLDIACETAMIDE

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The inhibition of glutamate oxidation by various fluorene carcinogens was reported in a brief communication (1). Of the pyridine nucleotide-linked oxidations studied in rat liver mitochondria, L-glutamate, α -ketoglutarate, *d*-isocitrate, and DL- β -hydroxybutyrate, only glutamate oxidation was markedly inhibited. The degree of this inhibition was roughly correlated with the known carcinogenicity of the fluorene compound (2). Further investigations on the nature of the inhibition are presented in this paper.¹ The results of these studies indicate that the carcinogen, *N*-2-fluorenyldiacetamide,² inhibits the glutamic dehydrogenase of mitochondria by acting as a powerful inhibitor of DPN⁺ for the enzyme. In this respect, the "native enzyme" present in mitochondria appears to be distinctly different from crystalline glutamic dehydrogenase, which is only slightly inhibited in a non-competitive manner by the carcinogen.

Materials and Methods

DPN⁺ and DPNH of 90 per cent purity, cytochrome *c*, and crystalline adenosine 5'-phosphate were commercial products. The fluorene compounds were generously contributed by Dr. H. P. Morris and Dr. H. Dyer, *d*-isocitrate was kindly supplied by Dr. G. H. Hogeboom. Crystalline glutamic dehydrogenase was a product of C. F. Boehringer, Mannheim, West Germany.

Oxygen uptake was determined by the usual Warburg manometric method, and inorganic phosphate by the Fiske-Subbarow method. Mitochondria were isolated from rat liver by the isotonic sucrose method of Schneider (4), with slight modification (5). Extracts of mitochondria were prepared by suspending the particles, washed three times, from 5 gm. of liver in 20 ml. of 0.07 M KCl-0.025 M K phosphate buffer, pH 7.6, by freezing, thawing, and homogenizing them two times, and finally by centrifuging

¹ A part of these studies has been published in a preliminary note (3).

² Abbreviations used in this paper are as follows: 2FdAA, *N*-2-fluorenyldiacetamide, DPN⁺, diphosphopyridine nucleotide, DPNH, reduced diphosphopyridine nucleotide.

the suspension at $110,000 \times g$ for 25 minutes in the Spinco preparative ultracentrifuge. The clear yellow supernatant extract containing glutamic dehydrogenase was kept frozen until used. Crystalline glutamic dehydrogenase centrifuged from 1 ml of suspension in ammonium sulfate solution was dissolved in 5 ml of 0.2 M K phosphate buffer, pH 7.6, and thoroughly dialyzed against the buffer to remove ammonium sulfate. The enzyme was kept frozen when not used.

Glutamic dehydrogenase activity was usually determined from the rate of reduction of DPN⁺. The test system contained 0.2 ml of 0.3 M K phosphate, pH 7.6, DPN⁺ as indicated in Tables I to V and Figs. 1 to 3, 0.05 ml of enzyme diluted when necessary with 0.07 M KCl-0.025 M K phosphate, pH 7.6, 0.1 ml of 0.3 M K glutamate, pH 7.6, and water to make a final volume of 3 ml. When activity was determined in the presence of carcinogen, the carcinogen was added either as a dilute aqueous solution replacing most of the customary water volume or as an ethanolic solution, usually 0.05 ml, in which case an equal volume of ethanol was added to the control cell. Ethanol addition was made after addition of enzyme to prevent precipitation of carcinogen at high levels, and, in these instances, glutamate was added last to start the reaction. When aqueous solutions of carcinogen were employed, the enzyme was added last. The change in optical density at $340 m\mu$ for 2 or 5 minutes was taken as the activity reading. The initial reading for zero time was taken 30 seconds after the reaction was started. The method of Olson and Anfinsen (6) was used when glutamic dehydrogenase activity was determined by oxidation of DPNH.

Results

Experiments with Whole Mitochondria—The inhibitory effect of the carcinogen, 2Fd1AA, on the oxidation of a number of different substrates is shown in Table I. It is especially noteworthy that glutamate oxidation as well as the accompanying phosphorylation was almost completely inhibited by the carcinogen (87 per cent inhibition) in contrast to the other pyridine nucleotide-linked oxidations studied which were considerably less inhibited. The oxidation of *d*-isocitrate, presumably also involving a DPN⁺-linked oxidation in mitochondria (7, 8), was moderately inhibited (37 per cent) and that of β -hydroxybutyrate a little less so (27 per cent). The oxidation of α -ketoglutarate was only slightly inhibited (10 per cent), while that of succinate was almost unaffected. Inhibition of oxidation was in each case accompanied by lower phosphate uptakes which were proportional to the decreased oxygen uptakes. The P/O ratios were thus not influenced by the carcinogen.

The degree of inhibition of glutamate oxidation by 2Fd1AA appeared to be influenced by a number of factors. The effect of carcinogen concentra-

tion, the "state" of the mitochondria, and addition of DPN^+ on the inhibition response is shown in Table II. It is seen that, at an external carcinogen concentration of 1.2×10^{-4} M, inhibition of oxidation became significant. Furthermore, the inhibition increased after the mitochondria had stood for 30 minutes in the cold, although in this time interval the oxygen uptake of the uninhibited system remained unchanged. Finally, it is

TABLE I

Inhibition of Oxygen and Phosphate Uptakes by N-2-Fluorenyldiacetamide in Oxidation of Various Substrates by Rat Liver Mitochondria

Substrate	Oxygen uptake		Phosphate uptake		P/O	
	Control	2Fd1AA	Control	2Fd1AA	Control	2Fd1AA
	μl	μl	μmoles	μmoles		
L-Glutamate	129	17	24.9	2.0	2.17	1.32*
D-Isocitrate	106	67	17.2	11.0	1.82	1.84
DL- β -Hydroxybutyrate	61	45	15.1	11.5	2.77	2.86
α -Ketoglutarate	88	79	17.6	16.6	2.24	2.35
Succinate	269	264	31.9	30.8	1.33	1.31

* Phosphate uptake in this case was too small for accurate measurement. In other experiments in which less inhibition was noted, the P/O ratios determined for glutamate oxidation in the presence of 2Fd1AA were equal to those of the controls. The reaction vessels contained 0.05 M histidine, 0.005 M MgCl_2 , 0.0167 M phosphate, 0.0069 M adenosine 5'- PO_4 , 1.33×10^{-5} M cytochrome c, 0.038 M KCl, and oxidizable substrate at the following concentrations: 0.035 M glutamate, 0.01 M isocitrate, 0.015 M α -ketoglutarate, 0.015 M β -hydroxybutyrate, 0.04 M succinate, all were added as the K salt adjusted to pH 7.0, 0.025 ml of absolute ethanol with or without dissolved carcinogen, 1.5×10^{-4} M 2Fd1AA final concentration, and 0.1 to 0.2 ml of mitochondria and water. Final volume, 1.5 ml, pH 7.0. Incubation time, 30 minutes, including 5 minutes for equilibration, temperature, 28° . Oxygen uptake during the equilibration period was assumed to be equal to that measured during the first 5 minutes. To stop the reaction, 0.3 ml of 15 per cent perchloric acid was added from the side arm. The results are expressed according to the mitochondria obtained from 100 mg of liver.

noted that addition of DPN^+ in excess over the amount of carcinogen present prevented inhibition to a large extent.

In some early experiments on the inhibition effect, it was observed that mitochondria which had lost phosphorylating ability showed considerably less inhibition of glutamate oxidation by 2Fd1AA than did more intact particles. It was of interest therefore to see what effect 2,4-dinitrophenol would have on the inhibition response of intact mitochondria. In Table III it is seen that dinitrophenol appeared to release the inhibition caused by the carcinogen. From an initial inhibition of 94 per cent, when no dinitrophenol was present, the inhibition declined progressively with in-

creasing concentrations of dinitrophenol until, at 2×10^{-4} M dinitrophenol, the inhibition amounted to only about 13 per cent

The inhibition of glutamate oxidation by 2Fd1AA was found to be reversible. Mitochondria preincubated with 1.5×10^{-4} M 2Fd1AA showed no

TABLE II

Influence of Carcinogen Concentration, "State" of Mitochondria, and Addition of DPN⁺ on Inhibition of Glutamate Oxidation

Conditions	Oxygen uptake				
	2Fd1AA, 0.00	2Fd1AA, 6×10^{-5} M	2Fd1AA, 1.2×10^{-4} M	2Fd1AA, 1.8×10^{-4} M	2Fd1AA, 2.4×10^{-4} M
	μ l	μ l	μ l	μ l	μ l
Mitochondria immediately after preparation	64	49	26	21	15
Mitochondria 30 min after preparation	65	40	7	2	2
7.5×10^{-4} M DPN ⁺ present in incubation medium, mitochondria 60 min after preparation	91		54		46

The conditions were those indicated in Table I, time, 18 minutes

TABLE III

Release of 2Fd1AA-Induced Inhibition of Glutamate Oxidation by 2,4-Dinitrophenol

Dinitrophenol concentration M	Oxygen uptake		
	Control	2Fd1AA	Inhibition
	μ l	μ l	per cent
0.00	85	5	94
2×10^{-5}	86	17	80
5×10^{-5}	81	33	59
1×10^{-4}	76	50	34
2×10^{-4}	78	68	13

The conditions were those described in Table I. The results are expressed according to the mitochondria obtained from 75 mg of liver

impairment of glutamate oxidation after the carcinogen was washed out or after a 10-fold dilution showed no more inhibition than a non-treated control in the presence of 1.5×10^{-5} M 2Fd1AA

Experiments with Extracts of Mitochondria—In an attempt to define more precisely the mechanism of action of the carcinogen on glutamate oxidation, the reaction was studied with extracts of mitochondria. In Fig 1, A, the relation between glutamic dehydrogenase activity of mitochondrial extract and DPN⁺ concentration in the presence and absence of 2Fd1AA is shown,

the behavior of crystalline glutamic dehydrogenase under similar conditions is shown in Fig 1, B. It is at once apparent that the carcinogen inhibited the mitochondrial enzyme to a significantly greater extent than the crys-

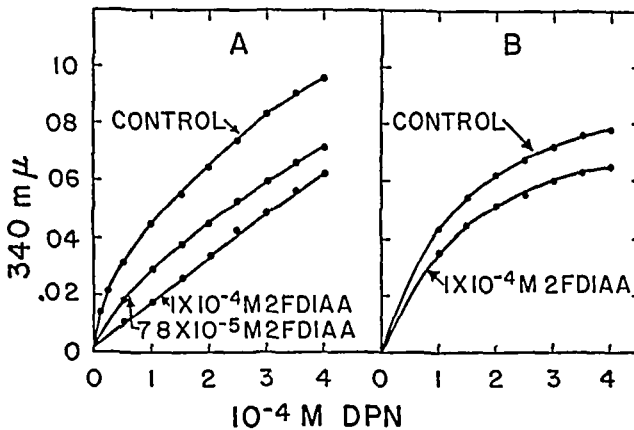


FIG 1 Relation between DPN^+ concentration and glutamic dehydrogenase activity in the presence and absence of 2FDIAA. A, mitochondrial extract, B, crystalline glutamic dehydrogenase. The reaction cell contained 2.5 ml of 1.52×10^{-4} M 2FDIAA to give a final concentration of 1×10^{-4} M carcinogen. Other additions and conditions are described in the text. Time, 2 minutes.

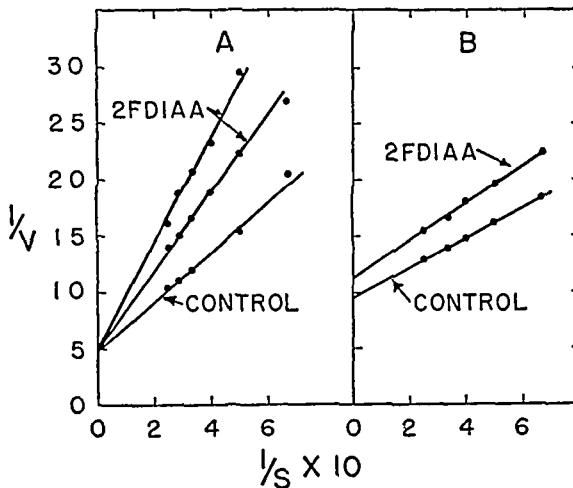


FIG 2 Lineweaver-Burk plot of the data in Fig 1. A, mitochondrial extract, B, crystalline glutamic dehydrogenase.

talline enzyme, particularly at the lower DPN^+ concentrations. A Lineweaver-Burk plot of the data of Fig 1, A, over a restricted range of DPN^+ concentrations is shown in Fig 2, A. The character of the plot indicates that a competitive type of inhibition is essentially involved. The slight in-

hibitory effect of the carcinogen on the crystalline enzyme appears to be non-competitive (Fig 2, B)

The effect of carcinogen concentration on inhibition of glutamic dehydrogenase activity is shown in Fig 3. It is seen that increasing concentrations of 2Fd1AA caused a progressive increase in inhibition to a point at which solubility of the carcinogen became the limiting factor, at about

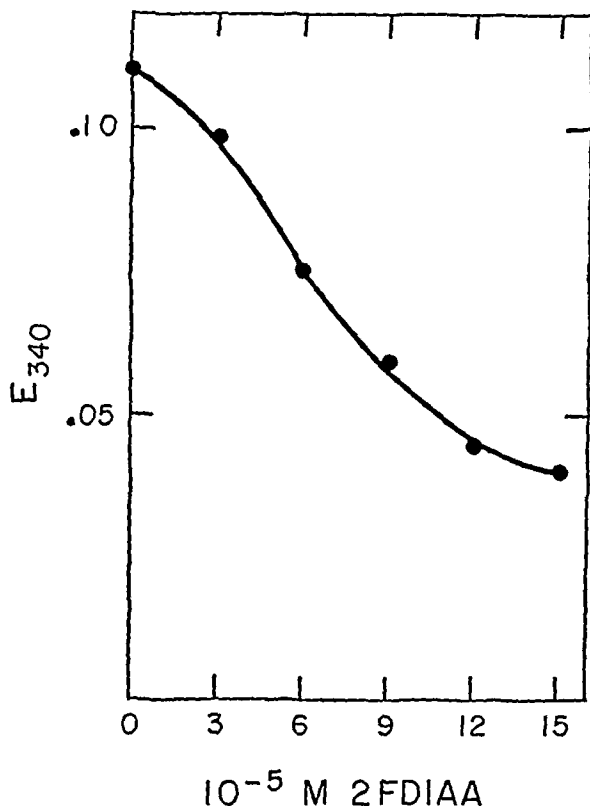


FIG 3 Effect of 2Fd1AA concentration on glutamic dehydrogenase activity of mitochondrial extract. DPN⁺ concentration, $6 \times 10^{-4} \text{ M}$, time, 2 minutes. 0.05 ml of ethanol containing dissolved carcinogen. Other additions and conditions were those described in the text.

$1.5 \times 10^{-4} \text{ M}$. It is also seen (Fig 3) that, under the conditions of the experiment (preincubation), 50 per cent inhibition occurred at a carcinogen to DPN⁺ ratio of about 1:6.

The effect of other fluorene compounds on the glutamic dehydrogenase activity of mitochondrial extract is shown in Table IV. *N*-2-Fluorenylacetamide was nearly as inhibitory as the corresponding diacetamide, whereas 2-fluoreneamine was somewhat less inhibitory. These compounds were followed by *N*-1-fluorenylacetamide and *N*-4-fluorenylacetamide in order of decreasing inhibition. Under the conditions of the experiment,

all of the fluorene derivatives tested exerted some inhibitory effect. Again, as in the experiments with whole mitochondria, there is a relation between the order of the compounds with respect to inhibition of glutamate oxidation and then relative carcinogenicity (1)

TABLE IV

Effect of Various Fluorene Derivatives on Glutamic Dehydrogenase Activity of Mitochondrial Extract

Fluorene compound	Glutamic dehydrogenase activity
None	0.098
N-2-Fluorenyldiacetamide	0.058
N-2-Fluorenylacetamide	0.060
2-Fluoreneamine	0.065
N-1-Fluorenylacetamide	0.071
N-4-Fluorenylacetamide	0.078

The cuvettes contained 0.02 ml of 0.009 M fluorene compound dissolved in ethanol, 0.05 ml of 0.015 M DPN⁺, 0.05 ml of mitochondrial extract, and other additions as described in the text, time, 5 minutes

TABLE V

Glutamic Dehydrogenase Activities of Mitochondrial Extract and of Crude Enzyme Fraction Precipitated by Ammonium Sulfate

Enzyme	Glutamic dehydrogenase activity			
	By DPN ⁺ reduction		By DPNH oxidation	
	Control	2Fd1AA	Control	2Fd1AA
Untreated extract	0.075	0.036	0.289	0.135
Crude enzyme fraction	0.054	0.032	0.167	0.128

Preparation of crude enzyme 5 ml of mitochondrial extract were treated with solid ammonium sulfate to 70 per cent saturation. The precipitate after 30 minutes was centrifuged, dissolved in 3 ml of 0.07 M KCl-0.025 M K phosphate, pH 7.6, dialyzed against repeated changes of the buffer to remove ammonium sulfate, and finally brought to the original volume of 5 ml with the buffer. 5 ml of untreated extract were dialyzed in the same buffer for the same period of time. *Conditions of assay* 0.05 ml of 0.015 M DPN⁺ or DPNH, 0.05 ml of mitochondrial extract or its equivalent, 0.025 ml of 0.009 M 2Fd1AA dissolved in ethanol, other additions and conditions were as described in the text, time, 5 minutes

Inhibition of glutamic dehydrogenase activity of mitochondrial extract by 2Fd1AA was independent of the direction in which the reaction was measured. As seen in Table V, at a DPNH concentration equal to that of DPN⁺, the percentage inhibition of DPNH oxidation was nearly the

same as that of DPN^+ reduction. Treatment of the mitochondrial extract with ammonium sulfate to 70 per cent saturation yielded a crude enzyme fraction which, after dialysis, showed less sensitivity to the inhibitory action of the carcinogen than the enzyme in the untreated extract, especially when the method of assay was by DPNH oxidation (Table V). The susceptibility of the isolated crude enzyme to the inhibitory action of the carcinogen appears to be intermediate between that of the native enzyme of the extract and that of the crystalline enzyme.

DISCUSSION

The effect *in vitro* of the fluorene carcinogens on biological oxidations in rat liver mitochondria appears to be an inhibition of DPN^+ -linked oxidations, notably glutamate oxidation. Studies on the mechanism of inhibition with soluble extracts of mitochondria clearly show that the carcinogen, *N*-2-fluorenyldiacetamide, acts as a powerful competitor of DPN^+ for the glutamic dehydrogenase of mitochondria. Depending upon the conditions of the experiment, at 50 per cent inhibition, the ratio of carcinogen to DPN^+ corresponded to values of 1.6 to 1.5, indicating that the inhibitor has an affinity for the enzyme that may be as great as or considerably greater than that of DPN^+ . The carcinogen functions as an effective inhibitor only on the native enzyme present in mitochondrial extract and not on crystalline glutamic dehydrogenase. There is some evidence from the decreased sensitivity of an isolated crude enzyme fraction that the isolation procedure in the preparation of the crystalline enzyme may bring about this change. This difference in sensitivity to the carcinogen of the "native" and the crystalline glutamic dehydrogenase may represent an example of the possibility that enzymes in the intact living cell may possess physiologically important properties which are not exhibited by the enzymes after they are isolated.

In considering possible reasons for the difference in the inhibition response between the glutamic dehydrogenase of mitochondrial extract and the crystalline enzyme, it might be supposed that the extract contained some material which, in association with the enzyme, was responsible for the diminished activity in the presence of carcinogen. No evidence was obtained, however, that material in the mitochondrial extract combined in this manner with added crystalline glutamic dehydrogenase. The inhibition observed with a mixture of the extract and the crystalline enzyme in the presence of carcinogen was the average of the inhibitions shown by the separate preparations.

The results of the present studies on the mechanism of inhibition of glutamate oxidation as carried out with soluble extracts of mitochondria offer some basis for the interpretation of the response of intact mitochondria to the carcinogen under a variety of conditions. In experiments

with whole mitochondria, it was observed that different preparations of mitochondria often showed considerable variation in their ability to oxidize glutamate in the absence of added DPN^+ and still more variation in the magnitude of the inhibition response to the carcinogen. Some preparations from presumably normal healthy rats would not oxidize glutamate at all without added DPN^+ , others would initially show some activity but would become inactive quickly (15 minutes in the cold). Dianzani (9) has observed similar instability of DPN^+ in liver mitochondria from animals receiving treatments that produce fatty livers. In general, mitochondria from adult male Sprague-Dawley rats, weighing about 200 gm and kept on a diet of Purina chow, were relatively stable, showing no decline in rate of glutamate oxidation in the absence of added DPN^+ up to 4 hours of storage at 0° . Such mitochondria were used in obtaining the results reported in this paper. In view of the dependence of the inhibition response on DPN^+ concentration, it is not surprising that mitochondria often showed these wide and changing responses to the carcinogen as a reflection of the state of DPN^+ within the particle. More specifically, the inhibition response might be directly related to the concentration of DPN^+ associated with glutamic dehydrogenase within the mitochondria. With so called tightly coupled mitochondria that maintain DPN^+ for relatively long periods, the degree of inhibitions observed was considerably greater than that frequently observed with less tightly coupled mitochondria that lose DPN^+ rapidly. The larger inhibition response of tightly coupled mitochondria, in spite of their expected higher total levels of DPN^+ , might be attributed to a rather limited supply of DPN^+ actually available to the enzyme, namely, that which is specifically bound to glutamic dehydrogenase and that which is present as free DPN^+ . The greater part of the DPN^+ in the mitochondria, viewed as being bound to other dehydrogenases (10), is therefore not available to afford protection against inhibition according to this picture. The release of the carcinogen-induced inhibition by 2,4-dinitrophenol, however, suggests that a relatively large amount of bound DPN^+ was set free by this reagent well known for its uncoupling effect on oxidative phosphorylation (11). The increase in free DPN^+ in the mitochondria by this postulated mode of action of dinitrophenol could provide considerable protection against the inhibitory action of the carcinogen. The state of DPN^+ in less tightly coupled mitochondria may be comparable to that produced by dinitrophenol in tightly coupled mitochondria. It is conceivable that, in the less tightly coupled state, the particles are not able to maintain DPN^+ in a bound condition very long, with the result that more free DPN^+ is available for a time (before undergoing enzymatic destruction or diffusion out of the particle) to provide some protection against inhibition.

The wide variation in the inhibition response of the same mitochondria

moved for duplicate whole blood CO_2 and O_2 content, O_2 saturation, and hematocrit determinations. The sample was centrifuged under mineral oil which had previously been saturated with CO_2 , and plasma was removed for the determination of CO_2 content in duplicate and pH in triplicate by gentle suction into a Van Slyke pipette. The remaining supernatant plasma and buffy coat were removed by suction into a micropipette and discarded. The red cells, still under oil, were hemolyzed without exposure to air by repeated freezing and thawing, by employing a dry ice-alcohol mixture. The red cell hemolysate was then analyzed for CO_2 and O_2 content in duplicate and pH in triplicate.

CO_2 and O_2 analyses of plasma, whole blood, and cell hemolysate were performed by the method of Van Slyke and Neill (7). The pH of plasma and cell hemolysate was determined with a Cambridge research model pH meter by employing a constant temperature ($37^\circ \pm 0.3^\circ$), water-jacketed MacInnes-Belchen glass microelectrode standardized to 0.01 pH unit after each measurement. Plasma water and whole blood water content were determined by drying known volumes to constant weight. Cell water content was calculated from these data and the hematocrit.

Calculations

It is assumed that the red cell is freely permeable to CO_2 and that the partial pressure of CO_2 in the plasma (P_{CO_2})_p is equal to the partial pressure of CO_2 within the red cell (P_{CO_2})_c. The value 6.11 is employed as pK'_1 for carbonic acid in plasma (3). No correction is made for carbamino protein in plasma. Total CO_2 (dissolved $\text{CO}_2 + \text{H}_2\text{CO}_3$) in the plasma and red cell, in milliequivalents per kilo of water, is obtained by multiplying the solubility coefficient of CO_2 , α , by P_{CO_2} (3), where $\alpha_p = 0.0334$, and $\alpha_c = 0.0362$. When all concentrations are in molal quantities (milliequivalents per kilo of water)

$$(1) \quad \text{pH} = \text{pK}'_1 + \log \frac{[\text{HCO}_3^-]}{[\text{CO}_2 + \text{H}_2\text{CO}_3]}$$

$$(2) \quad \text{pH} = \text{pK}'_1 + \log \frac{[\text{total CO}_2 - \alpha P_{\text{CO}_2}]}{[\alpha P_{\text{CO}_2}]}$$

Total CO_2 content of the red cell was corrected for carbamino hemoglobin at P_{CO_2} of 40 mm of Hg by assuming that 0.11 mmole of CO_2 combines as carbamino hemoglobin per mmole of oxyhemoglobin and 0.33 mmole of CO_2 as carbamino hemoglobin per mmole of reduced hemoglobin (5, 6). The partition of oxyhemoglobin and reduced hemoglobin in gm was calculated from the O_2 content and O_2 saturation, on the assumption that 1 gm of hemoglobin combines with 1.34 cc of oxygen (8). Plasma P_{CO_2} is

calculated from Equation 2 as

$$(3) \quad P_{\text{CO}_2} = \frac{[\text{total CO}_2]}{0.0334(\text{antilog}(\text{pH}_p - 6.11) + 1)}$$

and pK'_1 is calculated for the red cell from this value and the observed values for hemolysate pH and total CO_2 content as

$$(4) \quad \text{pK}'_1 = \text{pH}_c - \log \frac{(\text{total CO}_2 - \text{carbamino CO}_2 - 0.0362P_{\text{CO}_2})}{(0.0362P_{\text{CO}_2})}$$

Total CO_2 and O_2 contents of cell hemolysate check fairly well with the values calculated from whole blood and plasma analyses and hematocrit, but for our calculations we have preferred to use the latter because of the slow and variable drainage of the viscous hemolysate from pipettes

RESULTS AND DISCUSSION

Table I presents the data from which pK'_1 for carbonic acid in cells has been calculated. The average value for venous blood from eleven normal individuals is 6.18. In view of the total number of calculations required to obtain this final term, the range of the eleven values is not large (6.07 to 6.25). There is no overlap with the highest previous mean value, 6.04 (3), which was obtained by the examination of one sample of hemolysate examined at various CO_2 tensions and without correction for carbamino CO_2 .

Previous estimates of pK'_1 included the CO_2 in carbamino hemoglobin as part of $[\text{HCO}_3^-]$. As shown by Equation 1, any factor which reduces $[\text{HCO}_3^-]$, the other terms remaining constant, will increase pK'_1 , hence correction for carbamino hemoglobin operates to increase pK'_1 . The accuracy of this value depends in part on the accuracy of the constants defining the chemical combination of CO_2 as carbamino hemoglobin, and it must be noted that the constants used here are average ones calculated from the data of Ferguson and Roughton (5). We consider them as only approximations, since the method of measuring carbamino CO_2 is difficult and yields somewhat variable results.

Cell pH is a critical factor in calculating pK'_1 , and in our calculations the pH of the hemolysate is assumed to be identical with that of the intact red cells. This assumption is, we believe, warranted by Harris and Maizels' (9) demonstration that the buffering power of intact cells agrees closely with the buffering power of hemolyzed cells, indicating that rupture of the cell membrane does not alter cell proteins or buffers in a manner to affect $[\text{H}^+]$ significantly.

In non-ideal solutions, the activity of specific anions (or cations) differs from the total concentration in consequence of the restraining effects of

other ions This departure from ideal behavior is subsumed in the activity coefficient, γ , so that

(5) $[HCO_3^-] = \gamma[BHCO_3]$

The activity coefficient of $BHCO_3$ in body fluids is by custom incorporated with the dissociation constant, k_1 , and converted to the logarithmic ex-

TABLE I
Data from Which pK_1' for Carbonic Acid Is Calculated

Subjects	Whole blood					Plasma			Erythrocyte			Calculated pK_1' (corrected for carbamino CO ₂)	Calculated pK_1' (uncorrected for carbamino CO ₂)
	Hematocrit	O ₂ content	O ₂ capacity	CO ₂ content	Water content	pH	CO ₂ content	Water content	pH	Total CO ₂ content	Total CO ₂ carbamino CO ₂		
	per cent	cc per 100 cc	cc per 100 cc	m eq per l	per cent		m eq per l	per cent		m eq per l	m eq per l		
A D	47 8	15 2	19 8	22 2	82 0	7 41	26 9	92 6	7 26	17 1	14 1	6 17	6 08
M T	49 7	15 8	21 0	23 2	82 7	7 41	28 6	92 6	7 27	17 7	14 6	6 21	6 12
H R	42 8	11 4	18 0	24 4	85 2	7 40	29 3	92 6	7 23	17 8	14 3	6 21	6 11
A D	44 6	14 2	17 3	22 5	83 3	7 41	26 2	92 7	7 21	17 9	15 4	6 07	6 00
J B	47 6	14 5	20 7	22 0	82 3	7 39	27 6	92 6	7 16	15 8	12 4	6 16	6 05
P C	44 2	12 7	19 7	24 4	83 1	7 37	29 0	92 8	7 18	18 6	14 9	6 14	6 04
W F	44 1	9 7	18 8	24 2	83 3	7 42	29 2	93 5	7 22	17 9	13 7	6 16	6 04
A B	40 8	11 9	17 4	22 8	84 4	7 36	27 0	93 0	7 22	16 7	13 3	6 22	6 11
J B	50 0	13 3	22 3	22 7	81 6	7 40	28 6	93 2	7 25	16 8	12 9	6 24	6 11
F M	39 4	10 5	16 4	23 1	83 7	7 38	27 7	92 4	7 24	16 1	12 6	6 25	6 13
M S	43 2	8 3	16 8	22 0	83 6	7 39	25 5	93 2	7 22	17 4	13 6	6 14	6 03
Average												6 18	6 07

pression

(6) $pK_1' = \log \frac{\gamma}{k_1}$

and hence is concealed within pK_1' as calculated in Equations 2, 3, and 4

The activity coefficient, γ , is related to the total electrolyte content of the solution through μ , the ionic strength of the latter

(7) $\mu = 0.5 \sum C v^2$

i.e., μ equals one-half the sum of the terms obtained by multiplying the molal concentration of each ion, C , by the square of its own valence Debye and Huckel (10) demonstrated that, when the ions are treated as point charges at near infinite dilution, γ and μ have the following relation

(8) $-\log \gamma = \beta v^2 \sqrt{\mu}$

where v is the valence of the ion under consideration, and β is a constant expressing the effect of interionic forces. β cannot be determined directly for electrolyte solutions but is estimated to have a value of 0.522 at 38° for aqueous solutions of bicarbonate comparable to plasma.

With respect to k_1 , it will be recalled that this is only a nominal dissociation constant which treats all free, dissolved CO_2 as H_2CO_3 , whereas it is well known that equilibrium in the reaction



is such that the ratio of CO_2 to H_2CO_3 in aqueous solutions at ordinary temperatures is about 680:1 (11), i.e., the true first dissociation constant of H_2CO_3 is some 680 times the nominal value. That this distribution, as between CO_2 and H_2CO_3 , is identical in a cellular environment containing some 30 per cent protein and otherwise differing in composition from plasma is, at the moment, a purely gratuitous assumption, despite the fact that all concentration terms have been calculated per kilo of water. However, in the absence of evidence to the contrary, we assume that the apparent first dissociation constant, k_1 , of H_2CO_3 is the same in cells and plasma. It is further assumed that pH as measured by the glass electrode is related to $\alpha[\text{H}^+]$ in the same manner in the red cell and in the plasma.

The pK_1' for red cells (6.18) as calculated here is higher than that of plasma (6.11). If equilibrium distribution between CO_2 and H_2CO_3 is assumed to be the same in red cells and plasma, this difference may indicate a lower ionic strength in the red cell than in plasma. Alternatively, it may indicate that γ for HCO_3^- in the red cell is greater than that in the plasma, a circumstance contrary to the inference drawn by Van Slyke, Hastings, Murray, and Sendroy (2). Either situation may reflect the high concentration of protein in the cell. Further analysis must await a more accurate determination of the equilibrium relations in the formation of carbamino hemoglobin.

SUMMARY

pK_1' for carbonic acid in the red cell of normal human subjects has been determined after correction of total CO_2 content for carbamino hemoglobin. The average value for eleven individuals is 6.18 (range 6.07 to 6.25).

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SOME FACTORS INFLUENCING PHOSPHATIDYL- CHOLINE FORMATION*

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Conversion of ethanolamine to choline (1), with methionine serving as the methyl donor (2), has been established in the intact animal Stetten (1) observed that the labeled nitrogen of ethanolamine-N¹⁵ was retained in the choline molecule, and we determined that the carbon chain of ethanolamine is the precursor *in vivo* of the choline carbon chain (3)

Conversion of ethanolamine to choline by *in vitro* preparations is in a more doubtful state The synthesis of choline *in vitro* from methionine and ethanolamine with rat or guinea pig kidney and liver slices or homogenates (4, 5) has been questioned by Veritch and Zweig (6) These authors have demonstrated that the disappearance of methionine, as judged by the McCarthy-Sullivan method, was not the result of transmethylation but was actually due to the oxidative deamination of the methionine, mainly by D-amino acid oxidase present in the tissue preparations Similarly, the test for choline by precipitating it as the reineckate salt has been claimed to be unreliable, as ethanolamine and other bases that occur give insoluble reineckates (6) However, choline has been reported to be synthesized from L-methionine and 2-dimethylaminoethanol by rat liver slices in the presence of 0.001 M KCN, but not by liver homogenates (7)

Since the earlier *in vitro* experiments did not employ isotopic tracer methods and apparently did not utilize a reliable procedure for the isolation of choline, it appeared that the problem of the synthesis of choline from ethanolamine *in vitro* warranted reinvestigation In view of the lack of knowledge of the formation of the phosphatides, the present study of the metabolism of ethanolamine *in vitro* and the formation of choline was so organized as to permit the investigation to deal with the factors influencing the formation of lecithin or phosphatidylcholine

Methods

Non-fasted rats of the Long-Evans strain, weighing 165 to 175 gm, were killed by a sharp blow with the hand to the back of the head The liver

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or other tissues were immediately removed and placed in ice-cold physiological saline. Several slices totaling 500 mg of wet weight,¹ prepared with a Stadie-Riggs microtome, were incubated for 2 hours in 5 ml of Krebs-Ringer phosphate solution made up as described by Umbreit *et al* (8), with the exception that the CaCl_2 was 0.0016 M . The initial pH of the phosphate solution was 7.4. Incubation was carried out aerobically in 20 ml beakers at 37° with the Dubnoff apparatus (9). The rate of oscillation was maintained between 90 and 100 cycles per minute.

The radioactive substrates consisted of $3.8 \times 10^{-4}\text{ M}$ ethanolamine-1,2- C^{14} , prepared as previously reported (3), with 239,500 counts per 100 seconds per beaker, $3.0 \times 10^{-4}\text{ M}$ methionine- C^{14}H_3 with 311,000 counts per 100 seconds per beaker, and $3.0 \times 10^{-4}\text{ M}$ sarcosine- C^{14}H_3 with 185,000 counts per 100 seconds.

The reaction was stopped by the addition of 2 ml of a 3:1 mixture of ethyl alcohol-ethyl ether. In addition, to facilitate the denaturation of the proteins, the thermostat of the incubator was turned up so as to increase the temperature to 75 to 80° and shaking was continued for another 15 minutes. The denatured tissue was then extracted for phosphatides or stored in the deep freeze until ready for extraction. Extraction for phosphatides, hydrolysis, and the column chromatographic separation of the phosphatide constituents on Dowex 50 resin were carried out as described previously (3), with the exception that approximately 8 mg of carrier choline were added to the mixture of phosphatides and hydrochloric acid before hydrolysis.

The elution point (Ep) of choline from the Dowex 50 (250 to 500 mesh) resin column may be determined visually by examining the polyethylene collecting planchets for hygroscopic material. The Ep value has been arbitrarily defined in terms of the numbers of the planchets in which choline is found. This value is relatively constant if such conditions as the density and height of the resin bed, rate of flow and normality of the acid eluting liquid, and temperature are held constant.

The radioactivity of choline was determined directly in the planchets containing choline by employing the Tracerlab SC-16 windowless gas flow counter in conjunction with a Nuclear Instrument and Chemical Corporation scaler, model No. 163. In addition, the reneckate salt derivative was prepared by taking up the choline chloride in a minimal volume of water and adding to the aqueous choline 5 times its volume of a 2 per cent Reinecke salt-methanol solution. Choline reneckate was allowed to precipitate quantitatively by being stored in the refrigerator or the deep freeze for 6 hours. The precipitate was then plated on 4.25 cm Whatman No. 42 filter paper by use of conventional procedures, and counted for radioactivity.

¹The wet weight was taken after gently blotting each tissue slice on filter paper.

It should be pointed out that the resin column chromatography for the separation of choline assures the identity of the compound. No other compound known, which gives the reineckate salt reaction, possesses the same *Ep* value as choline. This is in contrast to the previous situation for the determination of choline reviewed by Jukes (10).

RESULTS AND DISCUSSION

The incubation of radioactive ethanolamine with rat liver slices in the absence of any cofactors resulted in a very small yield of phosphatidyl-

TABLE I

Effect of Factors on Formation of Phosphatidylcholine

Ethanolamine = 3.8×10^{-4} M, 239,500 counts per 100 seconds, methionine = 3.0×10^{-4} M, methionine- $C^{14}H_3$ = 3.0×10^{-4} M, 311,000 counts per 100 seconds, homocysteine = 3.0×10^{-4} M, cysteine = 3.0×10^{-4} M, betaine = 8.5×10^{-4} M, sarcosine- $C^{14}H_3$ = 3.0×10^{-4} M, 185,000 counts per 100 seconds

No of experimental results	Ethanol amine	Methionine	Homo cysteine	Betaine	Sarcosine	Cysteine	Total counts in phosphatidyl choline per 100 sec
6	+	0	0	0	0	0	98
7	+	+	0	0	0	0	1124
3	+	+	0	0	0	0	1928
4	+	+	0	0	0	0	2710
4	0	+	0	0	0	0	2800
5	+	0	+	0	0	0	586
4	+	0	0	0	0	+	98
8	+	0	0	+	0	0	196
3	+	0	+	+	0	0	804
2	+	0	0	0	+	0	99
2	+	0	+	0	+	0	170

* Radioactive

† 7.5×10^{-4} M methionine

choline,² representing about 0.04 per cent conversion (Table I). This was in contrast to the yield of phosphatidylcholine, representing 2.14 per cent conversion, obtained *in vivo* (3). Three possible explanations for the low yield were considered: (a) the 3 hour period of incubation was too long and most of the choline formed had been oxidized, (b) the radioactive choline found in the liver tissue of the intact animal may have originated in some other tissue, and (c) there was a relative absence of necessary cofactors or methylating substances. However, shorter periods of incubation of 30,

² The term phosphatidylcholine is used to describe the source of the radioactive choline since free choline is considered to be practically insoluble in petroleum ether (11).

60, and 90 minutes gave similar low values, as did incubations of tissue derived from spleen, kidney, intestine, and the pancreas

Effect of Methionine and Methionine- $C^{14}H_3$ on Phosphatidylcholine Formation—The addition of non-radioactive methionine, as a possible methylating substance, to the liver slices caused a maximal yield, under optimal conditions, of phosphatidylcholine, of about 19 times the basal level (Table I). The conversion under these conditions is approximately 0.8 per cent, which represents a considerable increase above the level of 0.04 per cent conversion, in the presence of ethanolamine alone, but still falls short of the 2.1 to 2.2 per cent conversion obtained *in vivo*.

The optimal concentration of methionine for the maximal yield of phosphatidylcholine under our conditions was 7.5×10^{-4} M, the use of higher concentrations of methionine either decreased the yield or exerted no effect. The ethanolamine substrate concentration apparently did not represent a limiting factor, as 65 to 70 per cent of the ethanolamine substrate always remained after the reaction was stopped.

As indicated in Table I, the transfer of the C^{14} methyl group of methionine to phosphatidylcholine is greater than the conversion of the C^{14} -labeled chain of ethanolamine on an equimolar basis.³ This increased yield of radioactive phosphatidylcholine, in the presence of labeled methionine, may of course be accounted for by the fact that, on an equimolar and random basis, for every labeled ethanolamine molecule there will be three labeled methyl groups entering into the reaction.

It will be noted that the presence of methionine is required for appreciable formation of radioactive phosphatidylcholine from labeled ethanolamine, whereas ethanolamine is not required for appreciable formation of radioactive phosphatidylcholine from labeled methionine.³ The observations of the low yield of phosphatidylcholine when labeled ethanolamine alone was incubated with liver slices and the necessity of adding methionine to the incubation of ethanolamine-1,2- C^{14} to achieve maximal phosphatidylcholine production indicate that the concentration of methionine available for methylation purposes in the rat liver slice is limited and that the liver slice contains a considerable metabolic pool of ethanolamine. This obser-

³ The results given in Table I indicate a slightly greater formation of choline from methionine in the absence of ethanolamine. In a more recent experiment performed by Mr. Akira Nakao, a 15 per cent greater increase in the label of choline (counted as the reineckate) was obtained in the presence of 2×10^{-3} M ethanolamine in the incubation medium than in its absence. In this same experiment it was determined that the methyl groups of choline contained over 90 per cent of the radioactivity when the radioactive substrate was methionine- $C^{14}H_3$. In earlier experiments with ethanolamine-1,2- C^{14} , it was found that about 20 per cent of the label appeared in the methyl groups of the choline. The methyl groups were cleaved as trimethylamine and measured as tetramethylammonium iodide.

vation is supported by the widespread occurrence of ethanolamine phosphate in tissues (12) and the regular excretion of ethanolamine in the urine (13)

Effect of Homocysteine, Cysteine, and Betaine on Phosphatidylcholine Formation—Homocysteine, as a precursor of methionine, would be expected to play an important role in the formation of phosphatidylcholine. The per cent conversion attained was 0.25, which is approximately 52 per cent of the yield procured in the presence of an equimolar concentration of unlabeled methionine (Table I). The action of homocysteine is intrinsic and not due to its SH group alone, since the presence of cysteine did not increase the average activity of choline above that of experiments in which ethanolamine-1,2- C^{14} was incubated alone.

Betaine was relatively ineffective as a precursor of methyl groups for phosphatidylcholine. The mean activity in phosphatidylcholine resulting from betaine was 34.5 per cent of the yield obtained from homocysteine. However, when homocysteine and betaine were added simultaneously to the substrate, the conversion of ethanolamine to phosphatidylcholine was as much as 4 times that obtained with betaine alone and nearly 2 times the yield obtained in the presence of homocysteine (Table I). The small effect of betaine on the formation of phosphatidylcholine *in vitro* and the synergistic action of betaine and homocysteine suggest that such a pool does not exist. At least it is not a pool in the sense that there is a transfer directly between these two particular members, *i.e.* choline and betaine, each participating equally. The transfer of deuterio methyl groups from betaine to choline (14, 15) may be explained by homocysteine serving as the carrier (16) for the betaine methyl groups. However, the function of the betaine methyl groups appears to require further investigation.

Effect of Homogenization on Formation of Phosphatidylcholine—Attempts to secure the methylation of ethanolamine to choline and phosphatidylcholine with rat liver homogenates in the absence and presence of adenosine triphosphate and folic acid were negative. The results were also negative when the homogenization was performed for periods as short as 30 seconds in a container surrounded by an ice bath.

Conversion in Vivo of Ethanolamine-1,2- C^{14} to Phosphatidyl Fatty Acids—After 4 hours, the magnitude of the conversion of ethanolamine to the phosphatidyl fatty acids, isolated from the whole liver of rat experiments *in vivo*, amounted to 0.015 per cent of the total injected activity (3 μ c).

The fatty acids contained in the residue from the filtration of the hydrochloric acid hydrolysate of the phospholipide were isolated and counted by redissolving in acetone and transferring the solution to flat metal cups. Upon evaporation of the acetone the cups were assayed for radioactivity.

Other Observations—Sarcosine- $C^{14}H_3$ showed a very small conversion of

0.05 per cent to phosphatidylcholine. The incubation of homocysteine with labeled sarcosine increased the yield of phosphatidylcholine about 70 per cent in two experiments. The interpretation of the increase in the yield of phosphatidylcholine in the presence of homocysteine is the same as with betaine, namely that homocysteine acted as a carrier of the sarcosine methyl group or more probably of the one carbon fragment derived from the methyl group of sarcosine.

The per cent conversion *in vitro* of ethanolamine-1,2- C^{14} (3.8×10^{-4} M, 239,500 counts per 100 seconds) to glycine was found to be 0.27 per cent, thus confirming the *in vivo* results reported by Weissbach and Sprinson (17). Glycine was isolated by adding 300 mg of carrier glycine to the *in vitro* mixture upon termination of the incubation. The preparation was then homogenized. After homogenization, ethyl ether was used to extract the 1 ml of 30 per cent trichloroacetic acid used to denature the incubation mixture. Following the extraction, absolute ethyl alcohol was added to precipitate the glycine. Glycine was crystallized twice from aqueous alcohol. The acetyl derivative of the recrystallized glycine was prepared, isolated, and degraded, and the glycine resulting from the degradation was then crystallized twice more before counting.

SUMMARY

In the absence of methionine, ethanolamine-1,2- C^{14} was not appreciably converted to phosphatidylcholine by rat liver slices. The addition of methionine increased the yield about nineteen times. The addition of methionine- $C^{14}H_3$ increased the yield of phosphatidylcholine about 3-fold above that obtained with unlabeled methionine. Evidence is presented which indicates that the methionine pool is much smaller than the ethanolamine pool. Homocysteine possesses 52 per cent of the activity of methionine, on an equimolar basis, in stimulating phosphatidylcholine formation, whereas betaine possesses only 34.5 per cent of the activity of homocysteine. Homocysteine and betaine together increased the yield of phosphatidylcholine considerably above the sum of the individual effects of each alone. The effect of homocysteine is intrinsic and is not due to the SH grouping alone.

Homogenization of the liver tissue resulted in complete loss of the ability to form choline. The conversion *in vivo* of ethanolamine-1,2- C^{14} to rat liver phosphatidyl fatty acids amounted to 0.015 per cent of the total injected activity. Sarcosine- $C^{14}H_3$ showed a small conversion of 0.05 per cent to phosphatidylcholine. The presence of homocysteine increased the yield about 70 per cent. The conversion *in vitro* of ethanolamine-1,2- C^{14} to glycine was 0.27 per cent.

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COEXISTENCE OF INSULIN-RESPONSIVE AND INSULIN- NON-RESPONSIVE GLYCOLYTIC SYSTEMS IN RAT DIAPHRAGM*

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Stadie and Zapp (1) have reported experiments on the aerobic production of lactic acid from glucose by the rat diaphragm *in vitro*, in which they used a phosphate-saline medium at pH 7.4, no bicarbonate being added. Under these conditions, the production of lactic acid was large but was not increased by the addition of insulin to the medium. This was in contrast to the observation that glycogen synthesis by these diaphragms was invariably increased by insulin. The increased synthesis of glycogen should be accompanied by a more rapid turnover of glucose 6-phosphate. However, this phosphate ester is the branch point of the Embden-Meyerhof system, and it would be expected that the formation of lactic acid would also be increased. Our early findings, therefore, appeared paradoxical, and stimulated us to project further experiments which might possibly illuminate this apparently anomalous observation of insulin action. Accordingly, in this paper, we report detailed experiments on lactic acid formation under the conditions given above. As before, we found a constant significant effect of insulin upon glycogen formation from glucose but no such effect upon lactic acid formation. From further experiments we conclude that there are two apparently identical enzymatic systems in the diaphragm, one of which is insulin-responsive, leading to the formation of glycogen, and the other insulin-non-responsive, leading to the formation of lactic acid.

Methods

Wistar male white rats (100 to 150 gm), fasted for 18 to 24 hours, were killed by cervical fracture, and the hemidiaphragms were removed, blotted, weighed, and washed for 10 minutes in a cold oxygenated medium without substrate. The hemidiaphragms were then placed in a small beaker containing 2 ml per hemidiaphragm of the following medium: 0.07 M NaCl,

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0.001 M CaCl_2 , 0.001 M MgCl_2 , 0.022 M glucose- U-C^{14} , and 0.050 M sodium phosphate (adjusted to pH 7.4 by a glass electrode). The specific activity of the glucose ranged between 16,000 and 22,000 c.p.m. per μmole of glucose. The beaker was shaken in the Dubnoff apparatus at 38° for 2 hours with oxygen as the usual gas phase. Paired hemidiaphragms from each rat were equilibrated (a) without insulin and (b) with 0.1 unit per ml of Lilly crystalline zinc insulin (0.04 to 0.05 per cent glucagon). When hexose phosphate esters were to be isolated, four hemidiaphragms were used per vessel.

Determination of Glycogen—The hemidiaphragm was washed, drained, placed in 5 per cent TCA overnight, and then ground with sand in a Potter-Elvehjem homogenizer. The suspension was centrifuged, and the supernatant fluid was decanted and saved for analysis of phosphate esters. The residue was resuspended in a small amount of 5 per cent TCA, centrifuged again, and digested at 100° in 30 per cent KOH. Glycogen was precipitated by adding 2.5 volumes of ethanol with chilling. The glycogen was precipitated three additional times to remove isotopic glucose, was hydrolyzed, and the glucose was determined by the Miller-Van Slyke method (2). An aliquot of the hydrolysate was plated, and from its C^{14} activity (open end gas flow counter) the specific activity was calculated.

Determination of Total and Specific Activity of Lactic Acid—A copper-lime filtrate of the combined medium and washes was prepared, and the total lactic acid was determined by the method of Barker and Summerson (3). An appropriate aliquot of the filtrate was plated and its C^{14} activity was determined (open end gas flow counter). From these two values the specific activity of the lactic acid was calculated. The assumption was made, from the following controls, that for the purposes of these experiments negligible amounts of radioactive derivatives of glucose other than lactate were present in the copper-lime filtrate. The most likely contaminants to be considered are pyruvate, malate, fumarate, succinate, and α -ketoglutarate. The first two at low concentrations may be removed by the copper-lime precipitation (3), an observation we have confirmed in the case of pyruvate. We tested this matter further by separating the lactate from the other possible contaminants by ascending paper chromatography with use of ethanol-ammonia (100:1 v/v). This method effects complete separation of the lactate. Controls showed that succinate, fumarate, and α -ketoglutarate remained at the origin, whereas the lactate moves 7 to 10 cm or more ($R_F = 0.3$ to 0.4). The lactic acid spot was localized by scanning with a closed end Geiger tube or by chromatographic comparison with a control spot of lactate sprayed with brom cresol blue. The spot was cut out and eluted with water, and the total lactic acid and radioactivity were determined as described. In a series of nine hemidiaphragms

equilibrated with isotopic glucose and treated as above, the specific activity of the eluate of lactic acid averaged 91 per cent of that calculated from the original copper-lime filtrate. At the beginning of the work, lactic acid was extracted from the medium by prolonged (Kutscher-Steudel) ether extraction, followed by chromatographic separation as described. The eluate of the lactic acid spot in sixteen experiments with rat hemidiaphragms averaged about 90 per cent of that of the initial ether extract. In no instance did insulin influence the relative specific activity of the lactic acid obtained by chromatography from that of the original material.

In view of these control experiments, we adopted the simpler method of using the copper-lime filtrate of the medium for the determination of specific activity of the lactic acid formed. Attention is called to the fact that the specific activity of the lactic acid is used only to calculate the percentage of lactic acid derived from the medium glucose. Since insulin never affected this value, possible errors of 10 per cent in its estimation have insignificant influence on the resulting general conclusions.

Determination of Pyruvic Acid—An ethyl acetate solution of the pyruvic 2,4-dinitrophenylhydrazone was prepared by the method of Friedemann and Haugen (4) from an aliquot of the combined medium and washes. The total amount of pyruvate present was determined colorimetrically (4) on an aliquot of the ethyl acetate solution. The remainder was evaporated to dryness (at about 35°) *in vacuo*, and residue was dissolved in 0.4 ml of ethanol and neutralized. The final volume was reduced to 0.1 to 0.2 ml by evaporation. The residue was placed on a 1.5 × 40 cm strip Whatman No. 1 filter paper in a band and hung for ascending chromatography in *tert*-butanol and ammonia (90:10 v/v) for 24 hours at room temperature. The spot corresponding to pyruvate was eluted with acidified ethyl acetate. A small aliquot (20 to 50 μ l) was used for the determination of C^{14} activity, and the remainder to determine the pyruvate present, the specific activity was then calculated. The most likely contaminating hydrazone is that of α -ketoglutarate, which is well separated from that of pyruvate by this procedure, *viz.*, the R_F value for the respective hydrazone is 0.22 for pyruvate and 0.68 for α -ketoglutarate. Unaltered 2,4-dinitrophenylhydrazine (little or none was present) remained at the origin. The spot was located by parallel chromatography of a known sample of pyruvic 2,4-dinitrophenylhydrazone.

Determination of Hexose Phosphate Esters, Barium Precipitation—The trichloroacetic supernatant fractions were combined with the washes from the four ground hemidiaphragms and adjusted to pH 8. 1 ml of barium acetate (0.1 M) and 4 volumes of ethanol were added and chilled for 30 to 45 minutes. The precipitate was centrifuged, washed once with 80 per cent ethanol, and then dried in air. It (barium salts of esters) was dis-

solved in 0.1 N HCl and shaken with Amberlite IR-120 (H form) resin (Rohm and Haas) to remove the barium ion. The solution of ester was drawn off, neutralized, and made up to 1.2 ml.

*Analyses for Total Esters, Glucose 1-Phosphate*¹—This ester is calculated from the difference between the initial inorganic phosphate and the 10 minute acid-hydrolyzable phosphate (1 N HCl at 100°). The micro-method of Dyer and Wrenshall (5), accurate for 0.1 to 0.01 μ mole of inorganic phosphate, was used.

Fructose 1,6-Diphosphate—In the presence of Racker's enzyme fraction II (6) (containing aldolase, triosephosphate isomerase, and α -glycerophosphate dehydrogenase), F-1,6-P is quantitatively converted to dihydroxyacetone phosphate, which is then reduced by added DPNH. The measured change (D) in optical density at 340 m μ gives the total F-1,6-P present, *viz*, F-1,6-P (micromoles) = $0.242 \times D$. The DPNH was prepared from DPN by use of alcohol and alcohol dehydrogenase (7).

Glucose 6-Phosphate—Two spectrophotometric methods were used. Under standard conditions, a TPN-G-6-P dehydrogenase system oxidizes G-6-P to 6-phosphogluconate. The measured change (D) of optical density at 340 m μ gives the initial micromoles of G-6-P, *viz*, G-6-P (micromoles) = $0.484 \times D$.

Racker's enzyme fraction I (6) (containing phosphohexoisomerase and phosphohexokinase) converts G-6-P to F-1,6-P. Together with Racker's fraction II, both G-6-P and F-1,6-P are completely converted to dihydroxyacetone phosphate, which is then reduced by the added DPNH. The total amount of G-6-P and F-1,6-P initially present is calculated from the observed change in optical density at 340 m μ , *viz*, total esters (micromoles) = $0.242 D$. G-6-P is calculated by subtracting the known F-1,6-P determined as above. Eluates of G-6-P obtained after chromatographic separation contain no F-1,6-P, hence, correction for the presence of F-1,6-P in this instance is unnecessary.

In general, amounts of phosphate esters were used for analysis to give changes of optical densities greater than 0.1. As little as 0.04 μ mole of the esters was determinable with an over-all accuracy of about ± 5 per cent. These enzymatic methods were carefully controlled for accuracy and specificity by analyses of known amounts of esters singly and in combination.

Chromatographic Separation of Hexose Phosphate Esters and Determination of Their Specific Activity—All chromatographic procedures were carried out at 0° for 16 to 18 hours by the ascending technique. Known sam-

¹ The following abbreviations are used in this paper: G-1-P, glucose 1-phosphate, G-6-P, glucose 6-phosphate, F-1,6-P, fructose 1,6-diphosphate, TPN, triphosphopyridine dinucleotide, DPN, diphosphopyridine dinucleotide, DPNH, reduced DPN, ATP, adenosine triphosphate, ADP, adenosine diphosphate, ATPase, adenosinetriphosphatase, and TCA, trichloroacetic acid.

ples of the phosphate esters were chromatographed simultaneously to locate the respective esters, the sharpest separation being obtained with 0.1 to 0.2 μ mole in about 20 to 50 μ l. The solutions were applied to the paper and dried with a heat lamp and fan. The Whatman No. 1 filter paper used had been washed with (a) 0.1 N acetic acid, containing a trace of Versene to remove traces of iron, and with (b) distilled water, and then dried for use. The R_F values observed by us (Table I) are different from those reported by Bandurski and Axelrod (8), presumably owing to the use of different paper, etc.

Glucose 1-Phosphate—The solvent mixture of Bandurski and Axelrod (8), methanol, formic acid, and water (80:15:5 v/v), was used to separate

TABLE I
*Chromatographic Separation of Hexose Phosphate Esters**

Compound	R_F	
	Acid solvent	Alkaline solvent
Glucose	0.45	0.69
Glucose 1-phosphate	0.30	0.39
Glucose 6-phosphate	0.43	0.38
Fructose 1,6-diphosphate	0.39	0.00
Inorganic phosphate	0.74	0.17

Whatman No. 1 filter paper was used after washing with 0.1 N acetic acid, containing a trace of Versene, and with distilled water. The paper was hung for ascending chromatography and run for 16 to 18 hours at 0°. The acid solvent consisted of methanol, formic acid, and water (80:15:5 v/v). The alkaline solvent consisted of methanol, ammonia, and water (60:30:10 v/v).

* Modified procedure of Bandurski and Axelrod (8).

G-1-P from G-6-P and F-1,6-P. After 18 hours the paper was dried and sprayed with a slightly modified Wade-Morgan (9) spray (ferric chloride and sulfosalicylic acid). The esters appear as two white spots on a pink background, and each was cut out and eluted with 1 to 2 ml of water. The G-1-P eluate was treated with Amberlite IR-120 resin (H form) to remove the iron. A 0.025 to 0.05 ml aliquot was plated to measure the C^{14} activity with an open end flow counter. The total G-1-P present was determined on another aliquot as described (see above), and the specific activity was then calculated.

Glucose 6-Phosphate and Fructose 1,6-Diphosphate—The eluate from the spot containing the mixture of G-6-P and F-1,6-P was treated with Amberlite IR-120 (H form) resin to remove the iron and then placed on the acid-washed Whatman No. 1 filter paper (see above). These esters were separated with a solvent system of methanol, ammonia, and water (60 -

30 10 v/v) (8) for 16 to 18 hours at 0° The dried paper was sprayed with the Wade-Morgan spray The respective spots were eluted with 1 to 2 ml of water and measured enzymatically as described Appropriate aliquots were plated for counting, and the specific activities were calculated It should be noted that glucose is not a contaminant since it is completely separated from the esters by the chromatography as well as the barium precipitation

Results

The data shown in Table II clearly indicate that the rat hemidiaphragm, when equilibrated *in vitro* in a medium containing glucose, synthesizes increased amounts of glycogen in the presence of insulin and that approximately 75 per cent of the insulin effect is accounted for by the increased conversion of medium glucose to glycogen In contrast, under the conditions of our experiments (see Table III), neither the total lactic acid nor the conversion of medium glucose to lactate is affected by insulin This is shown by the determination of both the total amount of lactate formed and that formed from the isotopic glucose 70 per cent of the total lactate formed is derived from the medium glucose, an amount unaffected by insulin

When pyruvate is isolated, the total production (2 to 3 μ moles per gm per 2 hours) and specific activity are not affected by insulin, but the specific activity is essentially the same as that of lactic acid

Isolation of the hexose phosphate esters, G-1-P and G-6-P, from the diaphragm, followed by their purification and determination of radioactivity, gave the results shown in Tables IV and V The amounts of these esters derived from the medium glucose are approximately doubled by insulin, indicating that their rates of turnover are increased by the hormone In striking contrast (Table VI), the fructose 1,6-diphosphate isolated from the diaphragm equilibrated with or without insulin never contained isotopic carbon This means that the G-6-P isolated from the diaphragms which always contained C^{14} in amounts increased by insulin (Table IV) was not the precursor of the F-1,6-P isolated from the diaphragm

In terms of the Embden-Meyerhof pathway of glycolysis, these results seem paradoxical It would be expected that the observed acceleration of the rate of turnover of G-6-P and G-1-P, accompanied by increased synthesis of glycogen, would be associated with an increased formation of F-1,6-P and hence also of lactate Accordingly, the C^{14} observed in the G-6-P should have been found in F-1,6-P However, this expected result was never observed under the conditions of our experiment

Additional experiments were devised to determine the possible occurrence of other departures of metabolism from that expected according to

TABLE II

Conversion by Normal Rat Diaphragm in Vitro of Glucose-U-C¹⁴ to Glycogen

Rat No	Final glyco gen, μ moles per gm per 2 hrs				Insulin effect, μ moles per gm per 2 hrs		
	Total		From glucose*		Total glyco gen	Glyco gen from glucose	Per cent total from glucose
	Insulin		Insulin				
	0	+	0	+			
7	72	96	18	37	24	19	79
8	57	75	10	29	18	19	105
9	29	42	6	13	13	7	54
10	32	49	12	25	17	13	77
46	22	35	4	10	13	7	54
47	18	37	3	17	19	14	74
Mean	38	55	9	22	17	13	74
S e m†	± 9	± 10	± 2	± 3	± 2	± 2	± 8

In Tables II to XII, the results are expressed in micromoles per gm wet weight of diaphragm. Glycogen is given as micromoles of glucose equivalents.

* Amounts of metabolites derived from glucose are calculated from total C¹⁴ activity of the metabolite and the initial specific activity (s a) of the medium glucose, e g, micromoles of glycogen from glucose = total counts per minute in glycogen divided by specific activity of glucose. The specific activity of the medium glucose usually ranged between 20,000 and 30,000 c p m per μ mole.

† In Tables II to XII, the standard error of the mean (s e m) is

$$\sqrt{\Sigma(X - \bar{X})^2 / N(N - 1)}$$

where N = the total number of observations

TABLE III

Conversion by Normal Rat Diaphragm in Vitro of Glucose-U-C¹⁴ to Lactic Acid at 38°

Rat No	Lactic acid, μ moles per gm per 2 hrs, recovered from medium					
	Total		From glucose		Per cent from glucose	
	Insulin		Insulin		Insulin	
	0	+	0	+	0	+
7	67	69	73	73	109	106
8	168	170	123	125	73	74
9	232	216	142	128	61	59
10	235	233	137	132	58	57
11	298	272	168	155	57	57
12	225	166	153	108	68	65
46	138	138	108	105	78	76
47	125	90	83	45	66	50
Mean	186	169	123	109	71	68
S e m	± 23	± 25	± 12	± 13	± 6	± 6

TABLE IV
*Incorporation by Normal Rat Diaphragm in Vitro of
 Glucose-U-C¹⁴ into Glucose 6-Phosphate*

Rat No	G 6 P from glucose U C ¹⁴			
	μ mole per gm per 2 hrs		Per cent total G 6 P	
	Insulin		Insulin	
	0	+	0	+
8	0 08	0 10	33	48
9	0 08	0 14	74	103
10	0 08	0 20	64	96
11	0 12	0 66	18	68
12	0 09	0 28	20	57
46	0 17	0 42	58	107
47	0 14	0 27	55	99
Mean	0 11	0 30	46	83
S e m	$\pm 0 02$	$\pm 0 12$	± 9	± 10

TABLE V
*Incorporation by Normal Rat Diaphragm in Vitro of
 Glucose-U-C¹⁴ into Glucose 1-Phosphate*

Rat No	G 1-P from glucose U-C ¹⁴			
	μ moles per gm per 2 hrs		Per cent total G 1-P	
	Insulin		Insulin	
	0	+	0	+
7	0 37	0 58	22	48
8	0 94	0 91	39	46
9	1 64	4 26	75	101
10	3 52	8 78	67	102
11	1 43	6 22	29	70
12	0 26	1 63	15	58
46	0 73	3 06	56	102
47	0 58	6 77	58	101
Mean	1 18	4 02	45	78
S e m	$\pm 0 96$	$\pm 1 07$	± 8	± 9

the conventional glycolytic concepts For example, certain non-isotopic intermediates were added to the medium together with isotopic glucose, and the effects on the isotopic lactate were studied

The results are shown in Table VII. Neither gluconate nor 6-phosphogluconate, intermediaries of the "direct oxidative" pathway and therefore outside the Embden-Meyerhof pathway, had any effect on the total lactic acid formed from the isotopic glucose of the medium, although the potential amounts of these available as lactic acid were many times greater than the lactic acid formed. In contrast, the Embden-Meyerhof intermediaries, pyruvate and 3-phosphoglycerate, were converted to lactic acid. This is shown by the increase of the total lactic acid, and by the dilution of the isotopic carbon in the lactic acid form. Anaerobiosis had no sig-

TABLE VI
*Incorporation by Normal Rat Diaphragm in Vitro of
Glucose-U-C¹⁴ into Fructose 1,6-Diphosphate*

Rat No	Fructose 1,6 diphosphate, μ mole per gm per 2 hrs, recovered from hemidiaphragm			
	Total		From glucose U C ¹⁴	
	Insulin		Insulin	
	0	+	0	+
7	0 39	0 42	0 00	0 00
8	0 49	0 70	0 02	0 01
9	0 65	0 65	0 07	0 09
10	0 63	0 68	0 03	0 03
11	0 70	0 81	0 00	0 00
12	0 51	0 57	0 00	0 00
46	0 11	0 13	0 00	0 02
47	0 14	0 12	0 00	0 00
Mean	0 45	0 51	0 02	0 02
S e m	$\pm 0 08$	$\pm 0 09$	$\pm 0 01$	$\pm 0 01$

nificant effect on the total lactic acid formed from glucose. Iodoacetate and fluoride, both of which are inhibitors of glycolysis, reduced the lactic acid formed from the isotopic glucose to very small values. These data show that the lactate was formed from glucose via the Embden-Meyerhof glycolytic cycle, by some mechanism which eliminates the formation of F-1,6-P in the diaphragm.

The situation was considerably clarified by the experiments reported in Table VIII. Diaphragms were equilibrated with non-isotopic glucose and isotopic G-6-P. The amount of the latter present in terms of lactic acid equivalents per gm was relatively small compared to the total lactic acid formed. However, about 10 per cent of the total lactic acid was derived from the G-6-P. In other words, an average of about one-third of the

phosphate ester was so converted. In contrast, none of the isotopic G-6-P appeared in the glycogen, although the customary increased synthesis from the medium glucose due to insulin was observed.

It began to be apparent that, under the conditions of our experiments, two separate pathways of glucose metabolism were operative in the rat diaphragm (1) to glycogen by way of G-6-P and G-1-P and (2) to lactic acid also by way of G-6-P. In the first instance F-1,6-P is not formed and

TABLE VII
Production of Lactic Acid by Rat Diaphragms in Vitro in Presence of Glucose-U-C¹⁴, Glucose Derivatives, or Glycolytic Poisons

Experiment No	Non isotopic addition			Lactic acid formed μ moles per gm per 2 hrs		
		Initial concentration, μ moles per ml	Lactic acid equivalents, μ moles per gm	Total	From isotopic glucose	Per cent total from glucose
1	None	0	0	222	118	53
	Gluconate	22	720	294	144	49
2	None	0	0	141	130	92
	6-Phosphogluconate	22	850	144	131	91
3	None	0	0	123	127	103
	Pyruvate	22	540	198	70	35
4	None	0	0	140	111	79
	3-Phosphoglycerate	5	97	206	97	47
5	Anaerobiosis			128	103	
6	Iodoacetate (aerobic)	1		9	8	
7	" "	0.1		56	14	
8	" (anaerobic)	0.1		29	17	
9	Sodium fluoride (aerobic)	20		17	13	
10	Sodium fluoride (anaerobic)	20		23	8	

G-6-P in the medium is not utilized, in the second instance, G-6-P in the medium, as well as glucose, is readily converted to lactate.

It is commonly stated that the hexose phosphate esters, *e.g.* G-6-P, in the extracellular medium do not enter the cells in experiments *in vitro*, and hence are not dissimilated. Presumably those formed in the cell do not readily pass from the cell interior to the medium. Accordingly, we formulated the following hypothesis to explain the results of our experiments thus far outlined.

The conversion of glucose to glycogen by the rat diaphragm is initiated by the transport of glucose to the interior of the cell, followed by esterifica-

tion and polymerization to glycogen. The phosphate esters formed, namely G-6-P and G-1-P, remain in the interior of the diaphragm. F-1,6-P is not formed from this internal G-6-P. Accordingly, no significant amount of lactic acid is formed within the interior of the diaphragm. In contrast, the conversion of glucose in the medium to lactic acid takes place in some other locus tentatively assumed to be the surface of the cell. The dissimilation of glucose follows the Embden-Meyerhof pathway. The phosphate esters formed on the cell surface do not enter the cell, nor do they accumulate in significant amounts in the medium. If they are added initially to the medium, however, they participate in the glycolytic reac-

TABLE VIII

*Formation in Vitro by Normal Rat Diaphragms of Lactic Acid and Glycogen in Presence of Non-Isotopic Glucose (0.022 M) and Glucose 6-Phosphate-U-C¹⁴ (0.002 M)**

Rat No	Lactic acid formed, μ moles per gm per 2 hrs				Final glycogen, μ moles per gm per 2 hrs			
	Total		From isotopic G 6 P		Total		From isotopic G 6 P	
	Insulin		Insulin		Insulin		Insulin	
	0	+	0	+	0	+	0	+
34	93	93	16 0	13 4	31	35	0 0	0 0
37a	139	91	7 3	5 4	38	47	0 0	0 0
37b	142	137	31 0	29 0	31	50	0 0	0 0
38a	106	89	4 8	3 8	24	33	0 0	0 0
38b	105	86	5 2	3 1	27	34	0 0	0 0
Mean	117	99	12 9	10 9	30	40		
S e m	± 10	± 9	$\pm 6 5$	$\pm 6 6$	± 2	± 3		

* The amount of G-6-P initially present was equivalent to an average of 32 μ moles of lactic acid per gm wet weight of diaphragm

tions and are converted to lactate. In addition to the data already presented, the experiments shown in Tables IX and X are completely compatible with this hypothesis.

In Table IX, the medium with which the diaphragms were equilibrated contained isotopic glucose and, in addition, non-isotopic esters. After 2 hours of equilibration, the medium, as before, was analyzed for lactate but, in addition, the phosphate esters were reisolated from the medium, purified by paper chromatography, and assayed for C¹⁴ activity. Compared to those of the controls, the data show the following: (1) in the case of G-6-P and F-1,6-P the total lactate formed was increased, the label from the glucose was diluted, and the reisolated ester acquired the label to an amount almost equal to that in the lactate formed from the isotopic

glucose, (2) in contrast, the medium G-1-P, when reisolated, contained no isotopic carbon Since our previous experiments showed that this ester is undoubtedly formed in the diaphragm from the isotopic glucose of the me-

TABLE IX

Equilibration of Normal Rat Diaphragms in Medium Containing Glucose-U-C¹⁴ (0.022 M) and Non-Labeled Phosphate Esters

Experiment	Final lactate in medium			Reisolated ester
	μmoles per gm per 2 hrs	C p m per μmole	Per cent total from glucose	C p m per glucose equivalent
No ester*	151	20,300	82	
G-6-P	200	17,200	70	15,800
No ester*	150	18,800	81	
F-1,6-P	177	14,100	49	16,000
No ester*	173	24,200	89	
G-1-P	161	23,000	84	0

* No ester glucose alone in the medium, the medium in the test in addition had phosphate ester equivalent to about 35 μmoles per gm wet weight of the hemidia-phragm

TABLE X

Conversion by Rat Diaphragm in Vitro of Glucose-U-C¹⁴ to Lactate, Glycogen, and Fructose 1,6-Diphosphate, Non-Labeled Fructose 1,6-Diphosphate Also in Medium*

	Total (μmoles per gm per 2 hrs)		μmoles from glu- cose (μmoles per gm per 2 hrs)		Per cent total from glucose		C p m per glucose equivalent	
	Insulin		Insulin		Insulin		Insulin	
	0	+	0	+	0	+	0	+
Lactate	128	102	109	65	85	64	8,700	6,540
Glycogen	10	16	3	8	30	50	2,870	5,150
F-1,6-P,† from me- dium							10,200	10,600
F-1,6-P, from dia- phragm	1 1	1 6	0	0			0	0
Medium glucose							10,600	10,600

* Mean values N = 3 in each category
† Reisolated after 2 hour equilibration

dium, it is clear that, when formed in the diaphragm, this isotopic ester is retained intracellularly and does not exchange with the non-isotopic ester in the medium

This compartmental isolation of hexose phosphate esters is clearly shown

by the data in Table X. Normal rat diaphragms were equilibrated with and without insulin with isotopic glucose plus F-1,6-P in the medium. At the end of 2 hours, lactate and glycogen were determined as before, but, in addition, the F-1,6-P was isolated both from the washed diaphragm and from the medium. After separation by paper chromatography, the specific activities of the glycogen, lactate, and F-1,6-P were determined. As hitherto, there was no insulin effect on the formation of total lactate or that derived from glucose. The synthesis of glycogen, as customary, was increased by the insulin. Strikingly, the F-1, 6-P recovered from the medium

TABLE XI

Glucose (0.022 M) in Medium Dissimilated to Lactic Acid by Diaphragm and Not by "Leached" Enzymes in Postequilibration Medium

Period		Contents of equilibrating vessel	Lactate synthesis in period, μ moles per gm, mean \pm s.e.m.		Final glycogen, μ moles per gm Mean	
			Insulin		Insulin	
			0	+	0	+
A	0-40	Diaphragm + medium	62 \pm 4.1	60 \pm 3.1		
B	40-80	" + fresh medium	44 \pm 2.0	40 \pm 1.8		
C	40-80	Medium from Period A	0 *	0 *		
D	80-120	Diaphragm + medium from Period C	27 \pm 7.2	33 \pm 5.6	12	20

Each hemidiaphragm was carried through the steps outlined above. The medium was analyzed for lactate at the end of each period and the glycogen of the diaphragm determined at the end of Period D. N = three separate assays in each category.

* Not significantly different from zero.

(see Table VIII) acquired the isotopic label from the glucose essentially to the same extent as that of lactate. The F-1,6-P recovered from the diaphragm contained no isotopic carbon. These results are in complete accord with the hypothesis formulated.

*Exclusion of Presence of "Leached" Enzymes in Medium*²—The possibility that enzymes in the diaphragm were leached out during the equilibration and were responsible for the phenomena observed was obvious. However, it was rigidly eliminated by the control experiments illustrated in Tables XI, XII, and XIII. Rat hemidiaphragms (Table XI) were equilibrated in the phosphate medium with glucose for 40 minutes, and the

² The medium obtained after a preliminary period of equilibration with the diaphragm is called the postequilibration medium.

medium then was analyzed for lactate. The lactate formed in the presence of the diaphragm, as hitherto found, was uninfluenced by insulin (Period A). The hemidiaphragms were then transferred to fresh medium (Period B) and more lactic acid was formed, but the medium from Period A equilibrated alone (Period C) showed no significant increase in lactic acid content, indicating clearly that no enzyme system existed in the postequilibration medium² capable of dissimilating the glucose to lactic acid. During Period D, the hemidiaphragm was placed in the postequilibration medium from Period C. Lactic acid was formed, showing that no inhibiting factors were responsible for the failure of lactate to form in Period C. At the end of the

TABLE XII

Hexose Phosphate Esters in Medium Dissimilated to Lactic Acid by Diaphragm and Not by "Leached" Enzymes in Postequilibration Medium

Period		Contents of equilibrating vessel	Lactate synthesis during period, μ moles per gm, mean \pm s e m, from	
			Glucose 6 phosphate (0.022 M)	Fructose 1,6 diphosphate (0.022 M)
	<i>min</i>			
A	0- 40	Hemidiaphragm + medium	55 \pm 3.0	72 \pm 9.6
B	40- 80	Medium from Period A alone	0 \pm 0	0 \pm 0
C	80-120	Hemidiaphragm + medium from Period A	27 \pm 6.0	51 \pm 10.0

With each of the substrates (five replicates), one hemidiaphragm was carried through the successive periods, A, B, and C. The hemidiaphragm was equilibrated in fresh medium during Period B but the medium was not analyzed. There was no glucose in any medium.

experiment, the diaphragms were analyzed for glycogen content, the usual insulin effect on glycogen synthesis was found.

Experiments of a similar type were carried on with two phosphate esters, *viz* G-6-P and F-1, 6-P, but no glucose in the medium. In Period A (Table XII) (0 to 40 minutes) the hemidiaphragm formed lactate from both G-6-P and F-1, 6-P. In Period B (40 to 80 minutes), the respective postequilibration media alone formed no additional lactate. The addition of the hemidiaphragm to the postequilibration medium in the subsequent period (Period C, 80 to 120 minutes) again resulted in lactate formation in both cases. As in the case of glucose alone, the data show that no enzymes capable of dissimilating these phosphate esters to lactic acid were leached out of the diaphragms during the initial period of equilibration.

In addition to these studies which demonstrated no formation of lactic

acid from glucose, G-6-P, or F-1,6-P by postequilibration media, we performed experiments to determine to what extent, if any, the glycolytic intermediary hexose phosphate esters are dissimilated in these media to products other than lactic acid. In our experiments the postequilibration media were prepared in two ways (1) the metabolite was present during the preliminary period, in which case no further addition to the medium was made at the beginning of the second period of equilibration, (2) the metabolite was absent during the initial period of equilibration with the diaphragm, but was added to the medium at the beginning of the second period. The results were the same by either method, and hence were pooled.

Our results are presented in Table XIII. The initial period of equilibration with the diaphragm and the second period were each 60 minutes.

TABLE XIII
*Action of Postequilibration Media from Rat Diaphragms on
Glucose or Glycolytic Hexose Phosphates*

Metabolite in medium	Products of dissimilation	Results of tests for enzymes in medium by specific methods
Glucose	None	No hexokinase*
Glucose 1-P	"	" phosphoglucomutase
Glucose 6-P	"	" phosphohexoisomerase
Fructose 6-P	"	" phosphohexokinase*
Fructose 1,6-P	Equimolecular mixture of the triose phosphates (50-60 μ moles per gm diaphragm per hr), no lactic acid	Aldolase, no triose isomerase or glyceraldehyde-3-phosphate dehydrogenase

Each category of data represents the mean value of six to eight assays

* Assayed after addition of ATP to medium

In all but one instance the mean value of the measured dissimilation of the metabolite being tested was not significantly different from zero, and hence is designated as "None" in Table XIII. In one instance a numerical value is given. The following points are to be emphasized: (a) The glucose concentration was unchanged during 60 minutes in the postequilibration medium and there was no lactic acid formation. The leaching of a complete glycolytic system is therefore excluded. Special assays with G-6-P dehydrogenase after the addition of ATP failed to demonstrate the formation of G-6-P. The presence of glucohexokinase is therefore excluded. (b) There was no dissimilation of G-1-P by the postequilibration medium. The ester was measured quantitatively by determining the reducing value of the medium before and after 10 minute hydrolysis in 1 N HCl at 100°. The presence of phosphoglucomutase is therefore excluded. (c) We observed no dissimilation of G-6-P (see "Methods" for quantitative measurement).

Phosphohexoisomerase in the medium is therefore excluded (d) No dissimilation of F-6-P was demonstrable when specific enzymatic methods already outlined in "Methods" were used to measure the ester This corroborates the evidence obtained with G-6-P that phosphohexoisomerase is absent from the postequilibration medium In addition, when ATP was added to the medium, no formation of F-1,6-P was observed (with Racker's enzyme fraction II (6) as outlined in "Methods") Thus, the presence of phosphohexokinase was excluded Finally, (e) F-1,6-P was rapidly dissimilated by aldolase leached into the postequilibration medium to an equimolecular mixture of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate This was established by using glyceraldehyde-3-phosphate dehydrogenase and DPN as an assay system (10) and observing a 1:1 molecular ratio between F-1,6-P and the glyceraldehyde 3-phosphate In addition, no lactic acid was formed Thus, the presence of both triose isomerase and glyceraldehyde-3-phosphate dehydrogenase in the medium is excluded The rate of dissimilation of F-1,6-P by the aldolase in the postequilibration medium, expressed in terms of the weight of the diaphragm present in the initial period, was found to be 50 to 60 μ moles per gm of diaphragm per 60 minutes

The presence of insulin in the medium was without effect in all of our experiments on the dissimilation of these metabolites by the postequilibration media

Zierler *et al* (11) and Beloff-Chain *et al* (12) have reported positive results in three instances in experiments on dissimilation of certain hexose phosphate esters by postequilibration medium from rat diaphragms In two instances their results are completely at variance with ours, in one instance there is agreement Zierler *et al* (11) reported that G-1-P was dissimilated to a mixture of G-6-P, F-6-P, and F-1,6-P These results indicated the presence of phosphoglucomutase, phosphohexoisomerase, and phosphohexokinase We never have been able to demonstrate any dissimilation of G-1-P and have ruled out the presence of all of these enzymes in our postequilibration media by specific enzymatic tests Beloff-Chain *et al* (12) reported the presence of phosphohexoisomerase in postequilibration medium dissimilating F-6-P or G-6-P to an equilibrium mixture Our experiments with these metabolites were completely negative Zierler demonstrated the presence of aldolase in his media in agreement with our results

No other data on dissimilation by postequilibration media were reported by these authors Beloff-Chain *et al* (12) compared the dissimilation of G-1-P and glucose with the diaphragm in the medium They found that G-1-P was dissimilated to a greater extent than glucose The addition of insulin, which customarily increases glucose uptake, actually decreased that of G-1-P The products of dissimilation were not recorded We

were unable to confirm these observations. In a series of experiments in which rat hemidiaphragms were equilibrated with 0.004 M G-1-P in the medium, we found no significant decrease in the ester after 60 minutes equilibration at 38° with oxygen as the gas phase.

We have no explanation for the discrepancy between our results and those of Zierler *et al* or Beloff-Chain *et al*. In several respects our techniques differed, in particular, our media always contained calcium (0.001 M) whereas the media of the above authors did not. Whether these technical differences can account for the discrepancies is a matter for future experimentation.

DISCUSSION

The experimental data outlined appear to us to demonstrate the following points: (1) Under the conditions of our experiment, *i.e.* use of a phosphate medium at a relatively high pH (7.4) with no added bicarbonate, the rat diaphragm *in vitro* can be shown to dissimilate the medium glucose by two different pathways. The intermediaries formed are those of the classical Embden-Meyerhof schema. The first pathway goes through G-6-P and G-1-P to glycogen. Apparently there is no formation of F-1,6-P from the G-6-P by this pathway. It is further characterized by being responsive to insulin, which causes a more rapid turnover of both G-6-P and G-1-P and a greater synthesis of glycogen from medium glucose. Furthermore, the phosphate esters formed, *i.e.* G-6-P and G-1-P, do not leave the interior of the diaphragm, nor do these phosphate esters, when added to the medium, enter the diaphragm to mix with the interior esters.

A second glycolytic pathway also forms G-6-P from medium glucose which is converted to lactic acid. However, the two G-6-P pools are not the same as shown by isotopic data. F-1,6-P is formed from this G-6-P formed by the second pathway, and is further dissimilated to lactic acid. The phosphate esters G-6-P and F-1,6-P, but not G-1-P, when added to the medium, readily enter into the metabolic system and interchange with the identical phosphate esters when these are formed from medium glucose.

(2) In no instance have we been able to demonstrate a dissimilation of medium glucose to lactic acid by any enzymatic system leached out of a rat diaphragm. Using specific enzymatic methods, we have carried out careful control experiments on media equilibrated with rat diaphragms. Under the conditions of our experiments we have demonstrated the complete absence in these media of the Embden-Meyerhof enzymes, glucokinase, phosphoglucomutase, phosphohexoisomerase, phosphohexokinase, triose isomerase, and glyceraldehyde-3-phosphate dehydrogenase. We therefore conclude that the phenomena reported cannot be accounted for by the presence of soluble enzymes in the medium leached out of the diaphragm.

The most plausible hypothesis which has occurred to us to explain these phenomena is to postulate the existence of two glycolytic systems which may be termed internal and external. The exact loci of these two systems need not be too closely defined. One may be considered to be intracellular and the other to exist upon the surface of the diaphragm. To attempt to define more closely the geometric localization of the surface enzyme would be pointless with our present knowledge of the phenomenon.

The possible existence of enzymes upon the surface of cells is not a new concept. Rothstein and Meier (13), using yeast cells, demonstrated an ATPase action by the cell surface. By using ATP labeled with P^{32} , their data excluded the possibility that the ATP was hydrolyzed within the cell, followed by a return of both the ADP and the isotopic phosphate into the medium. In addition, they excluded the existence of "leached enzymes," and furthermore showed that other dephosphorylating enzymes appeared to be functioning by the same mechanism. Marsh and Haugaard (14) have demonstrated an ATPase action on the cell surface of the isolated rat diaphragm which is not due to leached enzymes. Muntz, Singer, and Barron (15) concluded from experiments upon the glucose metabolism by yeast that the inhibiting action of uranium salts was at the surface of the cell. Rothstein and Larrabee (16) concluded from similar experiments with yeast that uranium existed at relatively high concentrations in an undissociated form upon the cell surface. They concluded that its presence at this locus inhibited oxygen uptake. This inhibition was reversed by the addition of small concentrations of inorganic phosphate. In contrast, the uranium inhibition observed in cell-free extracts of yeast was unaffected by phosphate. These authors cited these data as further evidence of the existence of active enzyme systems localized to cell surfaces. Sacks (17), in the study of the metabolism of injected isotopic phosphate by muscle, reported data from which he concluded that G-6-P is formed upon the cell membrane, followed by the penetration of the glucose portion to the interior cell and the subsequent hydrolysis of the phosphate linkage with the phosphate remaining in the extracellular phase. Lehninger (18) also invoked the existence of internal and external enzyme systems in mitochondria. He studied the oxidation of β -hydroxybutyrate, a DPN-linked substrate. Neither DPN nor cytochrome *c* need be added to the medium to obtain oxidation of the β -hydroxybutyrate. The internal DPN is reduced by the reaction and is reoxidized by the internal cytochrome *c*, the initial amounts of these factors present in the mitochondria suffice for the attainment of maximal rates of oxidation. This oxidation is accompanied by phosphorylation with a P/O ratio approximating 3.0. In contrast to this internal system, the oxidation of externally added DPNH requires the addition of cytochrome *c* to the test medium. "Clearly the externally added DPNH does not react with the same cytochrome *c* reductase molecule with

which the internal DPNH reacts" (18) The external oxidative system is not accompanied by any significant formation of energy-rich phosphate bonds, and furthermore antimycin A has no effect upon the external enzymatic reactions in contrast to its significant inhibition of the oxidation by the intact mitochondria These experiments appear to be the only ones we have encountered in the literature which demonstrate that enzyme systems essentially alike in their main features may coexist as internal and external systems

The experiments on the diaphragm reported in this paper appear to illustrate another example of this dual modality of enzyme action existing in one and the same tissue It appears to be unique in that the two systems have been shown to respond differently to a hormone In relation to current theories of insulin action, the data seem to be consistent with the hypothesis that insulin, at least in muscle, is concerned with transport of glucose across cell boundaries into the interior of the cell The glucose-glycogen system appears definitely to possess barriers which prevent the entrance or exit of phosphorylated sugars Since the diaphragm is also insulin-responsive, it might be assumed that it also possesses a barrier against glucose which is influenced by the hormone The glucose-lactic system, on the other hand, appears to permit free interchange of phosphate esters in the medium with "reactive" esters of the metabolic pool In other words, no barrier to the ready entrance of these substrates to the sites of enzyme action appears to exist Since the system is non-responsive to insulin, we may further assume that no barrier to glucose exists Although such evidence cannot be offered as proof of the transport theory, it seems to be more consistent with it than the hypothesis that insulin affects the hexokinase system This alternative would then require the assumption that two enzyme systems existing in one and the same tissue respond differently to a hormone This second alternative appears less likely than the first one

SUMMARY

Observations on the metabolism *in vitro* of hemidiaphragms from normal rats in a phosphate-saline medium (pH 7.4) + glucose- $U-C^{14}$ are reported Equilibration was usually for 2 hours at 38° with oxygen as the gas phase Total lactic acid formation from glucose was unaffected by insulin in the medium, in contrast, glycogen synthesis from medium glucose was always increased The Embden-Meyerhof intermediaries were isolated from the diaphragm and separated by chromatographic methods, and their C^{14} activity was determined These data show that insulin increases the turnover rate of glucose 6- and glucose 1-phosphates Surprisingly, C^{14} was never found in the fructose 1,6-diphosphate

Experiments with carrier or isotopic hexose phosphates in the medium,

together with glucose \pm insulin, were also performed. In some instances, upon equilibration, the esters were reisolated from medium and diaphragm. The results indicate that in the insulin-responsive (glucose to glycogen) system there is no interchange between esters in the medium and those in the diaphragm. In contrast, in the insulin-non-responsive (glucose to lactic acid) system there is a free interchange between the esters of the medium and those in the pathway of lactic acid formation. The possibility that enzymes leached out of the diaphragm into the medium are responsible for the results was excluded.

The experiments are compatible with the hypothesis that, under the conditions of our experiments, two glycolytic pathways are operative in the normal rat diaphragm: an internal (glucose to glycogen) system which is insulin-responsive, and an external (glucose to lactate) system which is insulin-non-responsive (surface). The data are in harmony with the concept that a transport mechanism in the diaphragm is involved in the insulin-responsive but not in the non-responsive system.

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OXIDATIVE PHOSPHORYLATION IN ACETOBACTER SUBOXYDANS*

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Oxidative phosphorylation has been widely studied over a period of years with respect to its distribution, its extent, and the mechanism of its operation. In animal systems, the outstanding features of the process include high P/O ratios and the coupling of much of the phosphorylation to the transport of electrons in the coenzyme or metalloprotein chain. In microorganisms, P/O ratios greater than 1 are occasionally observed (1, 2), but more frequently the values are less than 1 (3, 4), and the extent to which phosphorylation is coupled to electron transport is not known for many of these forms.

The present paper describes oxidative phosphorylation in intact *Acetobacter suboxydans* cells. Despite its aerobic characteristics, this organism appears essentially devoid of the Krebs cycle, the bulk of its energy being obtained from single step oxidations of carbohydrates and polyalcohols as well as from the pentose cycle (5, 6). The experiments to be described demonstrate phosphorylation coupled to oxidation of glucose, fructose, or glycerol, with low P/O ratios of 0.5 or less. Phosphate uptake is measured as the decrease in the concentration of orthophosphate within the cell and the medium. Pyrophosphate and polymetaphosphate may participate in phosphorylation in this organism.

Methods and Materials

A. suboxydans cells (ATCC 621) were grown in a yeast extract-glycerol medium, as described in earlier reports from this laboratory (7). The cells were suspended in phosphate buffer, 0.1 M, pH 6.0, and depleted for 2 hours.

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by being shaken at room temperature without a substrate. They were then washed twice in cold distilled water, and centrifuged at a standard speed to produce the wet cell paste used. The paste varied somewhat from batch to batch, but contained approximately 20 per cent dry matter. In the experiments in which phosphate consumption was measured, the cells suspended in malate medium (see below) were usually fasted for an additional hour before use.

The standard malate medium used in all the experiments consisted of 75 ml of 0.1 M malate buffer, pH 6.0, containing 0.001 M ethylenediamine-tetraacetate, 5 ml of 0.3 M $MgCl_2$, and water to 100 ml.

Inorganic orthophosphate was determined according to the Fiske-Subbarow method. Acid-labile phosphate was determined by heating the sample in 1 N H_2SO_4 in a boiling water bath for 15 minutes and determining orthophosphate as usual. Radioactivity in phosphorylated products (non-molybdate-reacting fraction) was determined according to Nielsen and Lehninger (8).

For the chromatographic separation of phosphate compounds, the method was that described by Hurlbert *et al.* (9), except that 8 per cent cross-linked Dowex 1 was used. The column was 20 cm long with a bed volume of 15 ml. The mixing flask contained 500 ml of distilled water and the reservoir flask contained 1 M ammonium formate, pH 5.0, at the beginning of the fractionation. 100.5 ml fractions were then collected, whereupon the contents of the reservoir flask were changed to 2 M ammonium formate, pH 5.0, and 70 more fractions were collected.

All radioactivity measurements were made upon 1 ml liquid samples in glass planchets and counted with an ordinary end window Geiger-Muller tube, to a precision of at least 2 per cent.

Carrier-free $H_3P^{32}O_4$ was obtained from the Oak Ridge National Laboratories, Oak Ridge, Tennessee.

EXPERIMENTAL

When resting cells of *A. suboxydans* are incubated aerobically in the presence of glycerol, oxygen is consumed at a rapid rate. The immediate product of this oxidation is DHA,¹ but oxygen is consumed to the extent of 4 to 5 atoms of oxygen per mole of glycerol, thus indicating that DHA is extensively oxidized. Fasted cells oxidize DHA very slowly, and it has been found in this laboratory that DHA must be phosphorylated before it

¹ The following abbreviations are used throughout this paper: DHA, dihydroxyacetone, TCA, trichloroacetic acid, ATP (A-R-P-P-P), adenosine triphosphate, ADP (A-R-P-P), adenosine diphosphate, P-P, inorganic pyrophosphate, GDP, guanosine diphosphate, CTP, UTP, and GTP, cytidine, uridine, and guanosine triphosphate, DPN and TPN, diphospho- and triphosphopyridine nucleotide, G-6-P, glucose 6 phosphate.

can be further metabolized (6). This shows indirectly that energy supplied by the oxidation of glycerol to DHA is used for phosphorylation, as the DHA is rendered available for metabolism by the simultaneous oxidation of glycerol.

Pure fructose is also slowly oxidized by depleted *A. suboxydans*. The oxidation may be started by the addition of 0.2 μ mole of glucose as a "sparker," then the oxidation proceeds at a steady and rapid rate. Once the oxidation has started, it will induce the phosphorylation of more fructose.

In order to demonstrate phosphorylation directly in *A. suboxydans*, it was necessary to use high cell concentrations and low concentrations of orthophosphate in the suspensions. In Fig. 1, Curve A, are given the results from a typical experiment with glycerol as substrate. When short time incubation was used, glycerol or glucose was chosen as substrate, on the assumption that there was no lag period in the oxidation of these substrates.

In some experiments, cell suspensions were incubated for 15 to 20 minutes with substrate, then the oxidation was stopped by filling the flask with N_2 or CO. Under these conditions, orthophosphate which was consumed during the aerobic phase of the experiment was rapidly liberated again. Fig. 1, Curve B, shows the results from this type of experiment. Fructose was used as substrate, because, after the initial lag, the oxygen consumption during the oxidation of this sugar is almost linear for a long period of time.

The final level of orthophosphate in a respiring cell suspension could be expected to represent an equilibrium concentration at which the orthophosphate consumption and liberation are equal in rate. The rate of phosphorylation might then be determined in two ways, either (a) directly by using short incubation times (small concentrations of phosphorylated products), thus diminishing the errors due to orthophosphate liberation, or (b) by interrupting the respiration and phosphorylation at equilibrium concentration of orthophosphate and measuring the initial rate of dephosphorylation (10). This method is based upon the assumption that the dephosphorylation proceeds at the same rate in the absence and presence of respiration. Data such as those shown in Fig. 1, Curve B, were analyzed in order to calculate the initial rate of orthophosphate formation. The rate of oxygen consumption was measured in parallel manometric experiments on the same cell suspension, and the data were used to determine the P/O ratios according to method (b) above. The values found with fructose as substrate were low, usually close to 0.2. It is uncertain whether gassing with N_2 or CO is effective in stopping respiration completely, and failure to do so might explain the low P/O ratios measured by this method as compared to those measured more directly (see below).

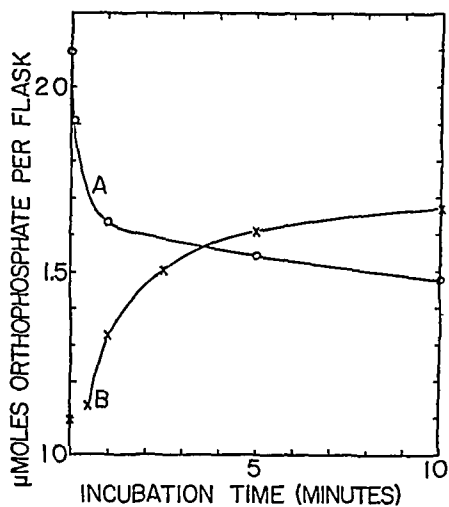


FIG 1

FIG 1 Curve A, the change in orthophosphate concentration with incubation time in a resting cell suspension of *A. suboxydans*-oxidizing glycerol. The suspension was made with 0.5 gm of wet cell paste per 20 ml in standard malate medium containing 0.1 ml of 0.1 M phosphate, pH 6.0, and was kept at 0°, and then 3 ml aliquots were removed, diluted with 0.5 ml of water, and equilibrated at 30° for 3 minutes with shaking. The reaction was started by the addition of 1.0 ml of 0.1 M glycerol, and stopped as desired with 0.5 ml of 50 per cent TCA. Substrate and TCA were added during shaking through flexible polyethylene tubes. Curve B, liberation of orthophosphate in a preincubated cell suspension after change to anaerobic conditions. Cell suspensions were made as described for Curve A. Each flask contained 3 ml of suspension, 0.5 ml of water, and 1.0 ml of 0.1 M fructose. The mixture was incubated aerobically for 20 minutes at 30°, at zero time was gassed with N₂ for 30 seconds, and the incubation was carried on anaerobically. The reaction was stopped by addition of 0.5 ml of 50 per cent TCA.

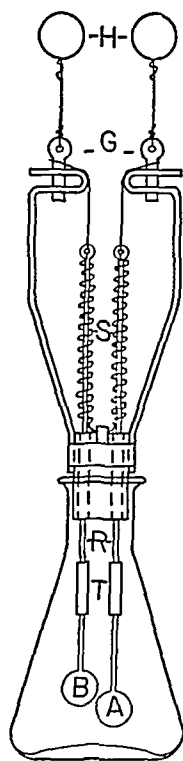


FIG 2

FIG 2 Incubation flask equipped for short time phosphorylation experiments. The glass bulbs, A and B, made from 3 mm outside diameter Pyrex tubing, are held on to the metal rods R by means of small diameter rubber tubing T. The stems of the bulbs are not closed after the contents are added, and the lower tapered part of rods R extend inside the stems of A and B, so that a rigid connection between glass and metal is obtained. The rods are fitted with springs S, which are held stretched by means of string loops and secured with the glass rods G. Pieces of aluminum wire H are used to remove G during shaking of the flask. When the springs are released in this way, the bulbs are broken and the contents of the bulb and the flask are mixed rapidly, due to the shaking. Additions can in this way be made within a few seconds, and the incubation times recorded with good precision.

Attempts were therefore made to use method (a) and to determine P O ratios by measuring the initial rate of orthophosphate disappearance. It became necessary to employ very short incubation times, and a special apparatus (Fig 2) was developed for this purpose. In these experiments no extra phosphate was added beyond that present in the depleted, washed cells themselves. To obtain sufficient precision in the determination of orthophosphate, it was necessary to use dense cell suspensions. During

TABLE I

Orthophosphate Consumption in A. suboxydans during Short Incubation Times with Glucose and Glycerol As Substrates

Substrate	Incubation time	Orthophosphate concentration	Average P consumption	P O ratio
	sec	μ mole per flask	μ mole	
Glucose	0 0	0 678		
"	3 2	0 588		
"	3 2	0 591	0 081	0 36
"	0 0	0 671		
"	3 0	0 594		
"	0 0	0 667		
Glycerol	0 0	0 681		
"	4 0	0 607	0 065	0 28
"	4 0	0 607		
"	0 0	0 663		

The cell suspension contained 2.5 gm of wet cell paste made up to 100 ml with the standard malate medium. 3 ml of the ice-cold suspension were equilibrated for 3 minutes at 30°, and then 0.5 ml of 0.1 M glucose or glycerol was added. 3 to 4 seconds later, 0.5 ml of 40 per cent TCA was added, the suspension was centrifuged in the cold, and orthophosphate was determined in the supernatant fluid. The additions of substrate and TCA were made by means of the apparatus shown in Fig 2, and the incubation time was measured with an ordinary stop watch. The suspension was diluted, 1:4, with the standard medium, and oxygen consumption, with the same volumes of suspension and substrate, was determined according to the conventional manometric technique.

the short incubation periods for which phosphate disappearance was measured, sufficient oxygen was present in the liquid so that overloading of the suspension with cells offered no difficulty. There was, however, difficulty in the separate, long time, manometric experiments in which the rates of oxygen consumption were measured, because these rates were 2 to 5 times higher than the oxygen diffusion rate under the conditions used. It was, therefore, necessary to dilute the suspensions in the manometric experiments and multiply the measured oxygen consumption values accordingly. The criticism may be offered against comparing the short period phosphorylations with longer period oxygen consumptions, in that a lag may

occur in the oxidation of the added substrates, and the observed P/O ratios would be spuriously low. However, as far as the limitations of the method would permit, higher rates of phosphorylation were observed as the incubation time was reduced.

By this method, the P/O ratios measured for glucose as substrate ranged from 0.31 to 0.41, and with glycerol from 0.27 to 0.35.

The protocol for a typical experiment is shown in Table I.

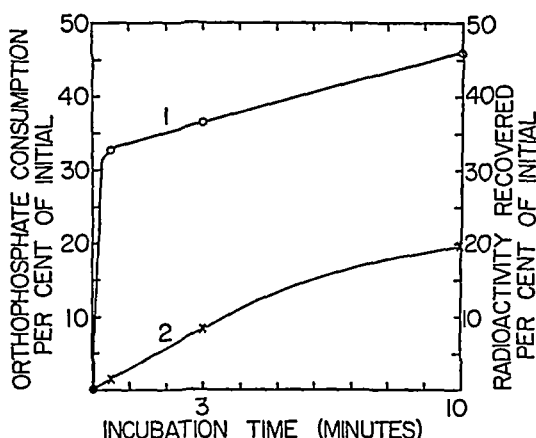


FIG. 3 Incorporation of orthophosphate and radioactivity into the non-molybdate-reacting fraction in *A. suboxydans*, during aerobic incubation with glycerol as substrate. Curve 1, consumption of orthophosphate, Curve 2, radioactivity recovered after removal of orthophosphate as the molybdate complex. Each flask contained 190 ml of suspension (12 gm of wet cell paste per 100 ml of suspension in standard malate medium. Carrier-free $P^{32}O_4^{3-}$ was added). A zero time sample was taken by adding 0.5 ml of TCA (1 gm per ml) to 9.5 ml of suspension. 1 ml of glycerol was added to each flask and the flasks were gassed with oxygen, and then shaken vigorously for 30 seconds, 3 minutes, and 10 minutes, respectively. The reaction was stopped by adding TCA to 5 per cent concentration. The suspension was cooled and centrifuged at about $15,000 \times g$ for 30 minutes. Aliquots of the clear extracts were analyzed for orthophosphate and non-molybdate-reacting radioactivity.

The phosphate consumption data in Table I were obtained by measuring relatively small colorimetric differences, and the error will therefore be large. An error not exceeding 25 per cent was estimated, based upon the accuracy of the colorimetric determination of orthophosphate and the range observed in the experiments.

Experiments with $P^{32}O_4^{3-}$ —A suspension of depleted and washed *A. suboxydans* cells was incubated for varying time intervals in an atmosphere of oxygen with glycerol as substrate in the presence of trace amounts of $P^{32}O_4^{3-}$. After TCA addition and centrifugation, aliquots of the clear extracts were analyzed for orthophosphate and for radioactivity in the non-molybdate-reacting fraction. The results are presented in Fig. 3.

To the remainder of the extracts, barium acetate was added to 2 per cent concentration, and sodium hydroxide to neutrality with phenol red. After at least 30 minutes in the cold, the precipitate was collected by centrifugation, washed with cold water, and shaken with Dowex 50 (hydrogen form)

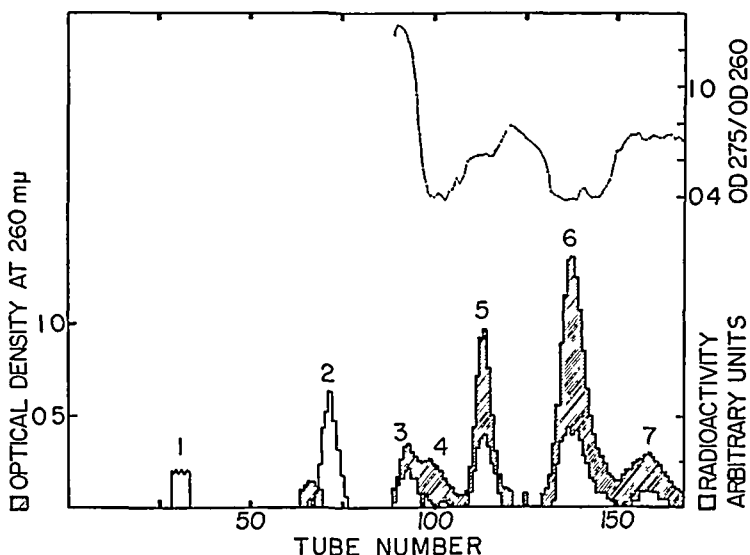


FIG 4 Ultraviolet absorption and radioactivity in the tubes from chromatographic fractionation of Ba-insoluble phosphates from the TCA extract of respiring *A. suboxydans*. The substrate was glycerol, and the incubation time 3 minutes. Trace amounts of P^{32} -orthophosphate were added to the medium. The curve enclosing the light areas corresponds to radioactivity in the respective tubes over the average background. The radioactive data were obtained with 0.1 ml samples from each tube and placed in a ruler-shaped sample holder, carrying forty samples, which was moved past the window of a Geiger-Muller tube connected to a recorder through a rate meter. In later experiments, the Geiger-Muller tube was placed with the window close to the collecting tube of a constant volume fraction collector, and the readings were recorded as before. When the tube was emptied, the reading would drop momentarily, indicating changing of tubes. The curve enclosing the shaded areas gives the optical densities at 260 $m\mu$ in the tubes, corresponding to the radioactive peaks. The points on the continuous curve to the upper right give the ratios for each tube between the optical density at 275 and that at 260 $m\mu$. The fractions indicated by numbers on the graph are Peak 1, inorganic orthophosphate (position only), Peak 2, inorganic pyrophosphate (partly overlapping ultraviolet-absorbing peak), Peak 3, CTP, Peak 4, ADP, Peak 5, UTP, Peak 6, ATP, Peak 7, GTP.

until dissolved. The resin was removed by filtration and washed, and the extract was again neutralized. The different phosphate compounds were then separated by ion exchange chromatography on a column of Dowex 1, formate form (9).

Successive samples of the tube contents were scanned for radioactivity, and the optical densities at 260 and 275 $m\mu$ were determined in the tubes corresponding to the radioactive peaks. Fig 4 shows the ultraviolet ab-

sorption and the radioactive data from a representative experiment. The ratios between the optical densities at 275 and 260 $m\mu$ identified the purine or pyrimidine part of the eluted nucleotides and indicated to what extent the fractions were overlapping, so that tubes containing pure substances could be used for further work.

Large amounts of strongly radioactive orthophosphate were eluted close to Tube 30 in most experiments. Around Tube 70 another strongly radioactive peak was eluted, which was identified as inorganic pyrophosphate. This was partly contaminated with another peak which probably contained a low concentration of a mixture of mononucleotides. Pyrophosphate was identified by means of paper chromatography in two different solvents: acetic acid-ethyl acetate-water, 3:3:1, and methanol-formic acid-water, 16:3:1. Also, after addition of carrier $\text{Na}_4\text{P}_2\text{O}_7$, it was repeatedly precipitated as the zinc salt at pH 3.8. The specific activities of the initial mixture, and after one, two, and three precipitations, were, respectively, 934, 775, 772, and 815 c.p.m. per μmole of acid-labile phosphate.

The following fractions were tentatively identified by means of their 275:260 ratios as CTP, ADP, UTP, ATP, and GTP. Occasionally small amounts of GDP were eluted after UTP.

Water and ammonium formate were removed from the pooled fractions. The nucleotides were conclusively identified and measured by their ultraviolet spectra (11, 12), and acid-labile phosphate and radioactivity were determined in all fractions. The results are given in Table II and Fig. 5.

The ATP from the 10 minute incubation period was allowed to react with glucose in the presence of hexokinase. ADP, G-6-P, and some AMP were isolated by direct chromatography of the reaction mixture. (G-6-P was identified and analyzed by its reduction of TPN with G-6-P dehydrogenase.) The data for ADP and AMP are included in Table II after correction for radioactive decay.

According to Fig. 3, the amount of radioactivity in the non-molybdate-reacting fraction was much lower, on a percentage basis, than the amount of orthophosphate that disappeared. The portion of radioactivity recovered approached one-half of the fraction of orthophosphate removed after 10 minutes of incubation. This has been confirmed in a number of experiments with incubation times up to 90 minutes. The same ratio of 0.5 was found, irrespective of whether extra orthophosphate or only trace amounts of $\text{P}^{32}\text{O}_4^{3-}$ were added to the medium. The disappearance of orthophosphate was initially a rapid process, but the incorporation of radioactivity into phosphorylated products reached its maximum much later. It is evident that the orthophosphate in the cell is preferentially used during the initial incubation period, and that some active respiration is needed before equilibrium is established between the phosphate in the medium and

that in the cells. Specific activity in the remaining total orthophosphate can be calculated. Since, after 30 seconds, the orthophosphate remaining (67.3 per cent) retained 98.6 per cent of the radioactivity, its relative specific activity would be $98.6/67.3 = 1.465$. The data calculated in this way

TABLE II

Relative Amounts of Acid-Labile Phosphate and Organic Base in Nucleotides Isolated from A. suboxydans and Specific Activity in Various Nucleotides

Incubation time	Nucleotide	Amounts isolated	Acid labile P groups per purine or pyrimidine	Specific activity
<i>sec</i>		<i>μmoles</i>		<i>c p m per μmole acid labile P</i>
30	UTP	1.79	2.04	4,720
30	ATP	2.45	2.00	5,470
30	GTP	0.53	2.47	5,540
<i>min</i>				
3	CTP	0.78	2.18	24,600
3	ADP	0.37	1.13	22,200
3	UTP	2.53	1.84	22,850
3	ATP	3.29	1.91	23,350
3	GTP	0.54	1.99	19,850
<i>min</i>				
10	CTP	0.84	2.11	53,200
10	ADP	0.13		55,800*
10	"	1.41	0.99	54,200†
10	UTP	1.75	2.14	45,300
10	ATP	3.83	2.17	45,800
10	GTP	1.23	2.02	42,400
10	AMP	0.26		9,030*†

Absorption spectra for the nucleotides were determined in 0.1 N HCl and 0.1 N NaOH, and the amounts were calculated.

* Specific activity given as counts per minute per micromole of purine.

† Isolated from the reaction mixture (ATP, hexokinase, glucose) after chromatography.

are plotted for comparison (Fig. 5, Curve 1) and coincide well with the experimental values.

In the same way the expected average of the specific activities of the products of phosphorylation was calculated. This was found to be lower than the activities of all the isolated fractions (Fig. 5) and indicated that orthophosphate of low activity had been incorporated into another phosphate compound, which was not isolated in this experiment. Such a compound might be high polymer metaphosphate, which has frequently been demonstrated in microorganisms (13, 14). It was found that part of the

phosphate consumed during oxidation of glycerol could not be recovered in the supernatant liquid (TCA extract) by analysis for acid-labile phosphate. Assuming that not all of the loss was due to the conversion into stable esters, part of the phosphate may have been bound in a fraction which is not soluble in 5 per cent TCA under the conditions of the experiment. This would correspond to the properties of metaphosphate (13).

A high polymer metaphosphate was later isolated from TCA-treated *A. suboxydans* by extraction of the residue with water at neutral pH (13). After centrifugation, the extract was treated with barium acetate, and the

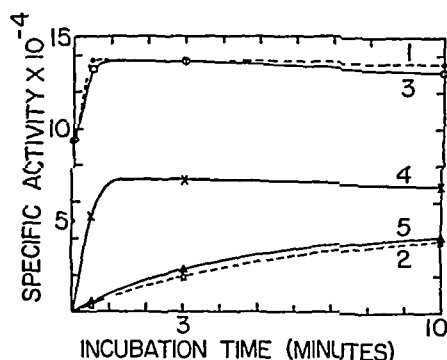


FIG 5 Change of specific activity (specific activity = counts per minute per micromole of orthophosphate or acid-labile phosphate measured as orthophosphate) with incubation time of the different phosphates in *A. suboxydans*. Curve 1, calculated activity of inorganic orthophosphate, Curve 2, calculated average activity of products of phosphorylation. Calculations were based on the data presented in Fig 3 (see the text), Curve 3, experimental data for orthophosphate, Curve 4, experimental data for pyrophosphate, Curve 5, experimental data for ATP (see Table II). The values refer to the acid-labile groups in ATP, and the data for the 10 minutes incubation period were corrected for activity in AMP. The specific activities in the acid-labile group in ADP and in those of the other isolated nucleotides were virtually identical with the corresponding activities of ATP.

precipitate was collected with centrifugation and rendered soluble with Dowex 50 (hydrogen form). The solution was clarified by centrifugation and reprecipitated with barium acetate at pH 3.0. After being rendered soluble with Dowex 50, the solution was dialyzed for 24 hours against distilled water, and then clarified by centrifugation when necessary. The solutions prepared in this way gave a purple to pink color with toluidine blue (30 mg per liter) and were hydrolyzed to orthophosphate in 1 N H_2SO_4 at 100° for 15 minutes. Some substances that absorbed in ultra-violet light were still present, but the calculated ratio between acid-labile phosphate and "purine" was more than 12:1.

The results from the time-course study (Fig 5) indicated that inorganic pyrophosphate might play some role as an intermediate product in phos-

phorylation in *A. suboxydans*. It was also suspected that metaphosphate might be of importance. These substances were therefore investigated for their possible ability to act as "sparkers" in the oxidation of fructose in fasted cells, some extracts, and the particulate preparation made from

TABLE III
*Oxidative Incorporation of Orthophosphate and Radioactivity
into Phosphate Compounds in A. suboxydans*

	Experiment 1		Experiment 2		Experiment 3	
	<i>per cent</i>	<i>specific activity*</i>	<i>per cent</i>	<i>specific activity*</i>	<i>per cent</i>	<i>specific activity*</i>
Orthophosphate consumption	31.2		28.2		30.2	
P ³² uptake, TCA-soluble fraction	3.5		11.4		15.1	
" " TCA-insoluble	0.0		2.5		3.4	
Orthophosphate						
Zero time		113,500		68,400		58,200
Calculated after incubation		109,500		82,000		67,800
Experimental from column		110,500		89,300		67,600
Pyrophosphate		55,900		38,200		28,500
ATP		10,150		29,600		25,600†
ADP						25,000†
AMP						14,700‡
Metaphosphate				2,800		1,060

The cell suspensions in all three experiments were 12 gm of wet cell paste per 100 ml of suspension in standard malate medium. The procedure was as in the time-course study. Experiment 1, the suspension was incubated for 15 minutes in oxygen with glycerol as substrate, but with no radioactivity. Then P³²-orthophosphate was added rapidly and the shaking was continued for 60 seconds more before the addition of TCA. Experiment 2, the suspension was incubated for 15 minutes in oxygen with fructose as the substrate and P³²-orthophosphate in the medium. Experiment 3, the suspension was incubated for 60 minutes in oxygen with glycerol as the substrate and P³²-orthophosphate in the medium. The values for the orthophosphate consumption and the P³² uptake are presented as per cent initially present.

* Specific activity = counts per minute per micromole of orthophosphate or acid-labile phosphate measured as orthophosphate.

† Corrected for radioactivity in AMP.

‡ Obtained as remaining activity after the acid-labile phosphate in ATP was removed by extraction as the molybdate complex.

alumina-ground cells described by Widmer *et al* (7). No such effects were found. The metaphosphate used in these experiments was prepared from *A. suboxydans* or made synthetically by heating KH₂PO₄ at 450° for 4 hours (15). During these experiments it was shown that large amounts of metaphosphate were associated with the particulate matter in the cells. A suspension of the particulates gave directly a pink to purple color with toluidine blue.

During respiration and phosphorylation in *A. suboxydans*, inorganic pyrophosphate was an early product as far as incorporation of radioactivity was concerned. It seemed of interest to examine this compound further with respect to its rate of isotope incorporation during oxidation of different substrates and over prolonged periods of incubation. Glycerol and fructose were selected as substrates, since the initial oxidation step for the former compound is independent of pyridine nucleotides, whereas the latter requires TPN or DPN (5, 6).

The results of these experiments, given in Table III, indicate that pyrophosphate had a high turnover rate, and reached its maximal level of radioactivity rapidly, even when the cells had been previously respiring for some time. After prolonged incubation (60 minutes) the radioactivity in ATP and ADP approached closely the activity in pyrophosphate or nearly one-half of that in orthophosphate. With glycerol as substrate, the activity in metaphosphate remained low even after 60 minutes of incubation.

The same pattern of incorporation of P^{32} was found with either fructose or glycerol as substrate. A considerable amount of radioactivity was, however, found in metaphosphate after only 15 minutes of incubation with fructose. This has been confirmed in several experiments. When excess glycerol was present as substrate, only small amounts of radioactivity were found in the insoluble (5 per cent TCA) sediment. When small amounts of glycerol were used, so that the oxygen consumption was greater than 1 atom of oxygen per molecule of glycerol or when fructose was the substrate, higher levels of radioactivity were found in the insoluble fraction.

Preliminary experiments with yeast showed that inorganic pyrophosphate was strongly radioactive after 5 minutes of incubation with ethanol as substrate, while the acid-labile phosphate groups of ATP and ADP had a much lower radioactivity (about one-seventh), but were equally active. Some P^{32} was found in metaphosphate.

DISCUSSION

The present experiments provide evidence of oxidative phosphorylation in intact *A. suboxydans* cells and demonstrate some important differences between phosphorylation in this organism and in animal tissues.

The P/O ratios during oxidation of glucose and glycerol in *A. suboxydans* are low, almost certainly less than 1. The phosphorylated products soon reach a maximal concentration, possibly due to lack of acceptors and because of the rapid dephosphorylation reaction described in yeast by Lynen and Konigsberger (10).

The nucleotides that are active in phosphorylation are present in low concentrations (approximately 1.5 μ moles of ATP per gm. of respiring cells, dry weight), and attempts to improve the yields of phosphorylated products through the addition of acceptors have been unsuccessful.

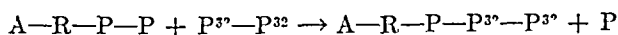
Inorganic pyrophosphate is present in relatively large amounts (about one-eighth of ATP) and turns over rapidly. However, its specific activity does not reach that of orthophosphate, but levels off close to half that value.

After prolonged incubation, the specific activities of the acid-labile phosphate groups in ATP and ADP approach that of inorganic pyrophosphate, not of orthophosphate.

Large amounts of high polymer metaphosphate are present in this organism, from 5 to 10 times the phosphate equivalent found in ATP. This material is slowly labeled by added $P^{32}O_4^{3-}$, although there is some evidence that metaphosphate is formed in resting cells during glycerol and fructose oxidation.

The rapid turnover of inorganic pyrophosphate in these experiments and the fact that the specific activities of the labile phosphates in ADP and ATP approach the specific activity of pyrophosphate rather than orthophosphate point strongly to inorganic pyrophosphate as an intermediate in oxidative phosphorylation in *A. suboxydans* as well as in yeast. This has been offered previously as a possibility in animal systems (16, 17), although the pertinent data would hardly serve to establish the point. Pyrophosphate was isolated from a rat liver preparation by Cori (18), and Lehninger and Smith (19) showed that radioactive pyrophosphate was formed in an active phosphorylating system containing $P^{32}O_4^{3-}$.

Of related interest is the fact that the two acid-labile phosphate groups of ATP are generally equally labeled, although this is not a universal observation (4, 20). When the two phosphates are equally labeled, as in the experiments of Krebs *et al* (21) and of Siekevitz and Potter (22), this has been ascribed to myokinase activity. Although this may explain many of the observations previously recorded in the literature, the *A. suboxydans* phosphorylating system would appear to operate differently. In this organism the concentration of AMP was small compared to that of ATP (5 per cent or less), and the rate of the myokinase reaction would have to be great in order to randomize the phosphate groups in ATP. A theory that would explain direct labeling of both acid-labile phosphate groups would seem to have some advantage over the addition of orthophosphate on to ADP. As ADP has been shown to be the preferred acceptor in phosphorylation in animal mitochondria (23), the following scheme may be suggested:



This scheme would confirm the observations in this paper and also those of Siekevitz and Potter (22) with unequally labeled $A-R-P-P^{32}$ as acceptor in mitochondria. These authors observed a great dilution of radioactivity in ADP and ATP, as well as the formation of radioactive orthophosphate inside the mitochondria during phosphorylation.

The *origin* of pyrophosphate, uncertain in animal systems, also remains to be explained in *Acetobacter*. The $P_2^{32}O_7^{4-}$: $P^{32}O_4^{3-}$ activity ratio of 0.5 may point to an interaction of 1 molecule of orthophosphate with a low activity P donor, possibly polymetaphosphate, to form a molecule of pyrophosphate. High polymer metaphosphate is present in large amounts in the particulate matter of the cells, in which the concentration of the respiratory pigments is high.

SUMMARY

Phosphorylation coupled to oxidation of glycerol, glucose, or fructose has been demonstrated in intact cells of *Acetobacter suboxydans*. P/O ratios are low (0.5 or less).

Time-course studies with $P^{32}O_4$ indicate that inorganic pyrophosphate may function as an intermediate in phosphorylation in this organism. The specific activity of pyrophosphate reaches approximately one-half that of orthophosphate.

Polymetaphosphate is found in relatively large amounts in *Acetobacter*. It is suggested that this material may participate in the formation of pyrophosphate.

The authors are grateful to Dr. C. H. Wang for supervision of the radioactive work, and to Dr. R. W. Newburgh for helpful advice.

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ENZYMATIC DECARBOXYLATION OF OXALIC ACID

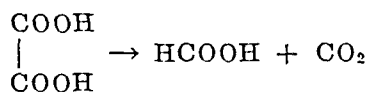
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Large quantities of oxalic acid accumulate in the cell sap of various plants and as a major product of carbohydrate metabolism by many molds. A recent investigation in our laboratory revealed that oxalic acid was produced from oxalacetic acid by an enzyme system which was isolated from the mycelia of *Aspergillus niger* (1). It was also demonstrated that oxalic acid was converted to formic acid and CO₂ by an enzyme isolated from a soil bacterium (2). The latter reaction proceeded anaerobically and required ATP,¹ CoA, Mg⁺⁺, ThPP, and acetate.

The present communication is concerned with the purification and properties of an enzyme which is obtained from the mycelia of a wood-destroying fungus, *Collyria velutipes*. This enzyme catalyzes the decarboxylation of oxalic acid with the formation of stoichiometric quantities of formic acid and carbon dioxide as follows:



In contrast to the oxalic decarboxylase of a soil bacterium, however, the enzyme from *C. velutipes* does not show a requirement for any of the cofactors mentioned above. Instead, it requires a small amount of oxygen, although the over-all reaction does not stoichiometrically utilize oxygen. Preliminary reports of this work have been published (3, 4).

EXPERIMENTAL

Manometric Method—CO₂ was determined by a conventional Warburg technique at 37°. Unless otherwise specified, the reaction was measured in an atmosphere of air with a 2 ml reaction mixture containing 400 μmoles of potassium citrate and 10 μmoles of potassium oxalate at pH 3.0. The reaction was started by the addition of the enzyme. 1 unit of activity was expressed as the amount of enzyme which causes the evolution of

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¹ The following abbreviations are used throughout this report: ATP, adenosine triphosphate, CoA, coenzyme A, ThPP, thiamine pyrophosphate, DPN, diphosphopyridine nucleotide, TPN, triphosphopyridine nucleotide

1 μ mole of CO_2 in a 1 hour period, and the specific activity was expressed in units per mg of protein. Protein was determined by the phenol method (5).

Anaerobic experiments were carried out as follows. 9 ml of a water solution, pH 3.0, containing 50 μ moles of potassium oxalate, and 2 mmoles of potassium citrate were saturated with helium gas² which had been passed through chromous chloride solution³. A 1.8 ml aliquot of this solution containing 10 μ moles of oxalate and 400 μ moles of citrate was placed in the main compartment of stoppered manometer flasks in which air had previously been replaced by helium. The enzyme solution and the other chemicals were added to the side arms of the manometer flask while it was being flushed with helium. Then the flasks were attached to the manometer and helium was passed through the entire manometer space for 10 minutes with shaking.

Spectrophotometric Assay—In order to determine the rate of the reaction with minute quantities of the substrate, the disappearance of oxalic acid was followed by determining at 25° the absorption at 220 or 230 $m\mu$ in a Beckman model DU spectrophotometer with quartz cells having a 1 cm light path. 50 μ moles of phosphate buffer, pH 3.0, were used in a total volume of 1.0 ml with 1 μ mole of oxalate and enzyme. Fig. 1 shows the ultraviolet absorption spectrum of oxalic acid at pH 3.0.

Determinations—Formic acid was determined by an unpublished method of J. C. Rabinowitz⁴. In the presence of ATP, formic acid, and an enzyme from *Clostridium cylindrosporum*, tetrahydrofolic acid is stoichiometrically converted to N^{10} -formyltetrahydrofolic acid. The reaction can be determined by the increase in absorption at 356 $m\mu$. The incubation mixture (1.0 ml) contained the following components, expressed in micromoles: maleate buffer, pH 7.0, 25; FeSO_4 , 0.5; MgCl_2 , 20; mercaptoethanol, 2.5; tetrahydrofolic acid, 1; ATP, 2. The mixture was incubated at 37° for 3 minutes, 0.1 ml of a partially purified enzyme preparation of *C. cylindrosporum* was then added and incubation continued for 10 more minutes. 1 ml of 5 per cent perchloric acid was then added, and the supernatant solution was used for the spectrophotometric determination.

Growth Conditions and Preparation of Crude Extract—*C. velutipes* (strain S) was grown on the surface of a medium containing 5 per cent dextrose, 1 per cent peptone, 0.1 per cent KH_2PO_4 , 0.05 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1 per cent Difco malt extract at pH 5.2. The medium (200 ml) was

² Grade A helium gas was obtained from the Bureau of Mines, Department of Interior, and was reported to contain less than 0.002 per cent (by volume) oxygen.

³ Adsorbent, chromous chloride solution, Burrell Corporation, Pittsburgh, Pennsylvania.

⁴ We wish to express our gratitude for the kind collaboration of Dr. J. C. Rabinowitz in carrying out the formic acid assay with us.

placed in Blake bottles of 1 liter capacity and incubated at 25°. About 25 days after inoculation, the reaction of the medium was brought to about pH 3.0 by the addition of about 2.5 mmoles of oxalic acid to each culture flask. The mycelia were harvested 2 to 3 days after the addition of oxalic acid. The culture medium was removed by filtration through a double layer of cheesecloth, and the mycelial pad was washed twice with cold distilled water and stored at -20°.

50 gm of frozen mycelia were ground in a Waring blender for 3 minutes with about 10 gm of crushed dry ice, and the fine powder was extracted with approximately 150 ml of 0.1 M potassium citrate buffer, pH 3.2, for 10 minutes at 4° with constant mechanical shaking. The suspension was

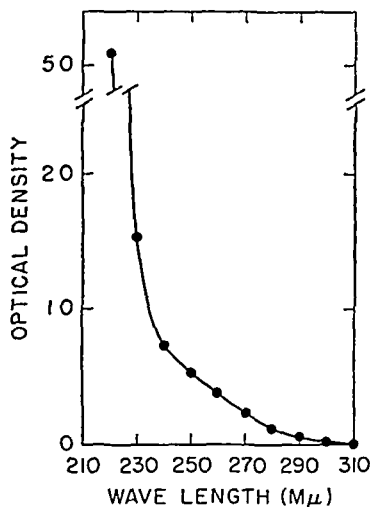


Fig. 1 Absorption spectrum of oxalic acid at pH 3.0 and 0.01 M

centrifuged at $10,000 \times g$ for 20 minutes at 0-2°. The supernatant solution thus obtained (crude extract) usually contained about 1.5 to 2.0 mg of protein per ml.

Material—Crystalline catalase was obtained from the Worthington Biochemical Corporation. α -Ketomalonate is a product of the H. M. Chemical Company, Ltd.

Results

Purification of Enzyme—All the manipulations were carried out at approximately 3°. Cold acetone (500 ml) was added dropwise to 1000 ml of crude extract with constant mechanical stirring. The precipitate was removed by centrifugation, and the precipitate formed upon further addition of 500 ml of acetone was dissolved in about 30 ml of 0.1 M potassium acetate buffer at pH 4.5 (first acetone fraction).

The first acetone fraction was dialyzed for 12 hours against 100 volumes of 0.02 M potassium acetate buffer at pH 4.5, and a small amount of precipitate formed during the dialysis was removed by centrifugation. To 10 ml of the supernatant solution were added 6.8 ml of cold acetone dropwise with constant mechanical stirring. The precipitate was removed by centrifugation, and 3.2 ml of cold acetone were added to the supernatant fluid in the same manner. The precipitate thus formed was collected by centrifugation and was dissolved in 2 ml of 0.1 M potassium acetate buffer at pH 4.5 (second acetone fraction).

The second step was repeated with the second acetone fraction, and the final preparation was dissolved in the same buffer (third acetone fraction).

The result of a typical purification is shown in Table I. Approximately 400-fold purification was achieved with an over-all yield of about 35 per

TABLE I
Purification of Oxalic Acid Decarboxylase

20 liters of culture (500 gm of wet mycelium)

Steps	Total volume	Units	Total protein	Specific activity	Yield
	ml		mg	units per mg protein	per cent
Crude extract	1600	30,720	3840	8	100
1st acetone fraction (33-50%)	50	22,100	110	200	72
Dialysis	53	22,400	101	220	73
2nd acetone fraction (40-50%)	10	12,700	5	2530	41.5
3rd " " (40-50%)	10	10,900	3.4	3166	35.5

cent. The specific activity of crude extracts varied from 8 to 73 among the six different batches, but the specific activities of the final preparations were almost identical and ranged from 3166 to 4040.

Stoichiometry and Cofactor Requirement—When 10 μ moles of oxalic acid were incubated under standard assay conditions in the Warburg manometer flask, 9.8 μ moles of CO_2 were produced and no measurable oxygen consumption was observed. After the reaction was over, the incubation mixture was removed from the flask and an aliquot was used for determination of formic acid, 10.3 μ moles of formic acid were produced. This result indicated that the over-all reaction was a stoichiometric decarboxylation of oxalic acid to form formic acid and CO_2 , and therefore it appeared to be identical with the enzymatic reaction previously described by Jakoby, Ohmura, and Hayaishi (2).

However, when the purified enzyme was dialyzed against cold distilled water for several days, there was little loss of activity, and the addition of CoA, ATP, Mg^{++} , acetate, or cocarboxylase, alone or in combination,

did not show any stimulation. When the enzyme was treated with Dowex 1 formate, the rate of the reaction was reduced to about 20 per cent of the original. However, the addition of either boiled crude extracts or ATP, CoA, Mg^{++} , acetate, and cocarboxylase did not increase activity.

These observations suggested to us that either the cofactors were bound extremely tightly to the enzyme protein or that the properties of the *Collybia* enzyme were different from those of the one previously described from a soil bacterium.

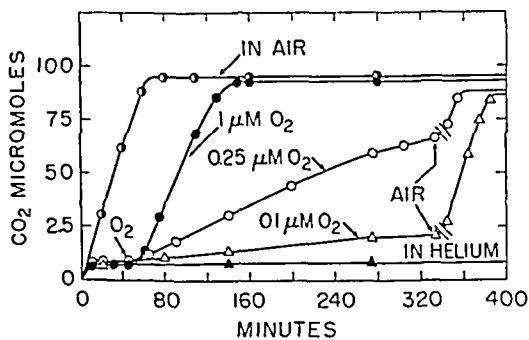


FIG 2

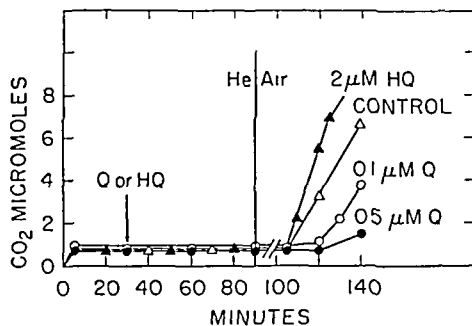


FIG 3

Fig 2 Effect of oxygen on the rate of enzymatic decarboxylation of oxalate. The incubation mixture contained, in 2.0 ml, 0.024 mg of enzyme protein (second acetone fraction), 10 μ moles of potassium oxalate, and 400 μ moles of potassium citrate buffer, pH 3.2. The branched side arm contained 10 units of catalase in one end and 2, 0.5, or 0.2 μ mole of H_2O_2 and 3 μ moles of potassium phosphate buffer, pH 7.0, in a total volume of 0.3 ml in the other end, temperature, 37°. The values were corrected for oxygen evolution.

Fig 3 Effect of quinone and hydroquinone on the rate of enzymatic decarboxylation of oxalate. The incubation mixture contained 0.024 mg of enzyme protein (second acetone fraction), 10 μ moles of potassium oxalate, and 400 μ moles of potassium citrate buffer at pH 3.2. Air was introduced at 90 minutes and exact readings were started at 105 minutes.

Effect of Oxygen on Rate of Reaction—It was previously observed that this reaction did not proceed under strictly anaerobic conditions and that introduction of air into the manometer flasks restored the activity to the original level (3). As shown in Fig 2, when the atmosphere was completely replaced by the helium gas (see under "Methods"), the gas production was almost negligible. In confirmation of the previously reported results, when air was introduced into the gaseous phase, the reaction proceeded immediately. In order to test whether oxygen is needed for the reaction, and how much oxygen is needed to restore 100 per cent activity, various amounts of oxygen were evolved in the manometer flasks as follows. A double side arm manometer flask was employed, one of the side

arms of which had two reservoirs (American Instrument Company, Inc., catalogue No 5-224) In one of these, 10 units of catalase were placed, and various amounts of hydrogen peroxide were placed in the other reservoir Usually the reaction was started by tipping in the oxalate solution from one side arm, and gas production was followed for about 30 minutes to make sure that the rate of the reaction was almost negligible Then the catalase and H_2O_2 were mixed in the branched side arm so that a known amount of oxygen was liberated in the manometer gas phase As shown in Fig 2, about 1 μmole of oxygen was necessary to restore the complete activity With 0.25 μmole of oxygen the effect was about one-fourth, and with 0.1 μmole of oxygen stimulation was about 10 per cent, of that observed with 1 μmole of oxygen In the absence of oxygen the enzyme

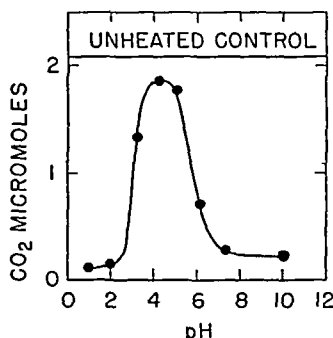


FIG 4 Heat stability of oxalic decarboxylase at various pH levels The enzyme solution (first acetone fraction) containing approximately 2 mg of protein per ml was heated at 78° for 10 minutes, cooled, and assayed with the standard assay system The pH was adjusted by the cautious addition of N KOH and H_2SO_4

appears to be completely stable, and even after 5 hours incubation the enzyme showed the original level of activity, once air was introduced

The requirement for oxygen was strictly specific and could not be replaced by any of the following compounds at concentrations of 0.01, 0.001, or 0.0001 M H_2O_2 , paraquinone, 2-methyl-1,4-naphthoquinone, flavin adenine dinucleotide, flavin mononucleotide, DPN, TPN, KNO_3 , and cytochrome *c* Hydroquinone and quinone exhibited no effect under anaerobic conditions, but when the air was introduced quinone exhibited a marked inhibition, as shown in Fig 3, whereas hydroquinone stimulated the reaction by 60 to 100 per cent Certain other reducing agents, such as catechol, ascorbic acid, and FeSO_4 showed some stimulating effect, but glutathione, cysteine, or cystine had no effect at 10^{-3} and 10^{-4} M Chelating agents such as ethylenediaminetetraacetic acid, KCN, and α, α' -dipyridyl (10^{-3} or 10^{-4} M) had no effect, nor did *p*-chloromercuribenzoate (10^{-4} M) have any inhibitory action on the most purified enzyme preparation

Stability—Crude enzyme solutions at various pH values were prepared by the cautious addition of HCl or KOH and heated at 78° for 10 minutes. The activity of these solutions was tested by the manometric technique. The enzyme lost only about 5 per cent of the activity at pH 4.0, but lost almost 90 per cent at pH 2.0 and 7.0 (Fig. 4). The crude enzyme preparation of pH 4.3 did not show any decrease in activity after storage at 0° for 45 days.

Influence of pH and Substrate Concentration—Maximal activity for the decarboxylation was observed in the range of pH 2.5 to 4.0 (Fig. 5). The

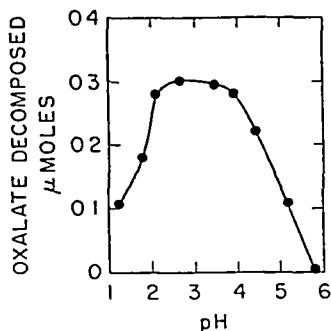


FIG 5

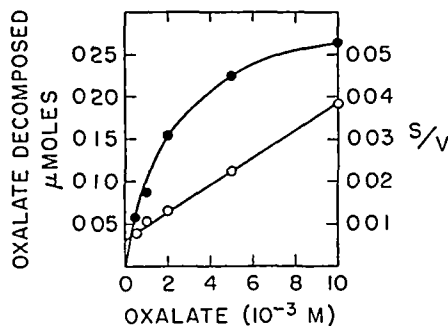


FIG 6

FIG 5 Effect of pH on the rate of the reaction. The reaction mixture contained 50 μmoles of potassium phosphate buffer, pH 3.0, 0.014 mg of enzyme protein (second acetone fraction), and 1 μmole of potassium oxalate in a total volume of 1.0 ml. Optical density measurements were taken at 220 mμ in a Beckman DU spectrophotometer for 3 minutes at 27°. The amounts of oxalate decomposed were calculated from the optical density of oxalate at each pH.

FIG 6 The rate of enzymatic decarboxylation as a function of substrate concentration (●). The conditions described in Fig. 4. Optical density was determined at 220 mμ or 230 mμ depending on the concentration of the substrate. The right-hand ordinate scale refers to a Lineweaver-Burk plot (6) of substrate concentration divided by velocity (S/V) as a function of substrate concentration (○).

influence of varying concentrations of the substrate is illustrated in Fig. 6. The apparent dissociation constant (K_m) of the enzyme and substrate is approximately 2.05×10^{-3} M.

Substrate Specificity—Oxalic acid was the only substrate found to be decarboxylated by the purified enzyme preparation. Pyruvic, malonic, succinic, glycolic, citric, malic, α-ketomalonic, oxalacetic, and formic acids were all inactive. The rigid specificity of this enzyme makes it an excellent tool for the specific and quantitative determination of oxalic acid.

DISCUSSION

It appears that there are at least three different enzymes which decarboxylate oxalic acid in nature. In higher green plants (6) and mosses

arms of which had two reservoirs (American Instrument Company, Inc., catalogue No 5-224) In one of these, 10 units of catalase were placed, and various amounts of hydrogen peroxide were placed in the other reservoir Usually the reaction was started by tipping in the oxalate solution from one side arm, and gas production was followed for about 30 minutes to make sure that the rate of the reaction was almost negligible Then the catalase and H_2O_2 were mixed in the branched side arm so that a known amount of oxygen was liberated in the manometer gas phase As shown in Fig 2, about 1 μmole of oxygen was necessary to restore the complete activity With 0.25 μmole of oxygen the effect was about one-fourth, and with 0.1 μmole of oxygen stimulation was about 10 per cent, of that observed with 1 μmole of oxygen In the absence of oxygen the enzyme

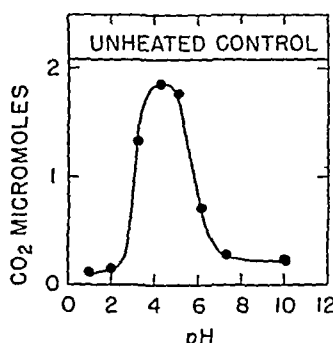


FIG 4 Heat stability of oxalic decarboxylase at various pH levels The enzyme solution (first acetone fraction) containing approximately 2 mg of protein per ml was heated at 78° for 10 minutes, cooled, and assayed with the standard assay system The pH was adjusted by the cautious addition of N KOH and H_2SO_4

appears to be completely stable, and even after 5 hours incubation the enzyme showed the original level of activity, once air was introduced

The requirement for oxygen was strictly specific and could not be replaced by any of the following compounds at concentrations of 0.01, 0.001, or 0.0001 M H_2O_2 , paraquinone, 2-methyl-1,4-naphthoquinone, flavin adenine dinucleotide, flavin mononucleotide, DPN, TPN, KNO_3 , and cytochrome *c* Hydroquinone and quinone exhibited no effect under anaerobic conditions, but when the air was introduced quinone exhibited a marked inhibition, as shown in Fig 3, whereas hydroquinone stimulated the reaction by 60 to 100 per cent Certain other reducing agents, such as catechol, ascorbic acid, and FeSO_4 showed some stimulating effect, but glutathione, cysteine, or cystine had no effect at 10^{-3} and 10^{-4} M Chelating agents such as ethylenediaminetetraacetic acid, KCN, and α, α' -dipyridyl (10^{-3} or 10^{-4} M) had no effect, nor did *p*-chloromercuribenzoate (10^{-4} M) have any inhibitory action on the most purified enzyme preparation

peroxide did not appear likely, although this hypothesis could not be ruled out completely

SUMMARY

Oxalic decarboxylase from *Collyria veltipes* was purified about 400-fold with an over-all yield of about 35 per cent. The enzyme is most active at pH 3.0 and most stable at pH 4.5. It acts specifically on oxalic acid and produces stoichiometric quantities of CO₂ and formic acid. In contrast to the previously reported oxalate decarboxylase from a soil bacterium, this enzyme does not show any requirement for adenosine triphosphate, coenzyme A, cocarboxylase, acetate, or Mg ion. However, it is active only in the presence of small amounts of oxygen, although the over-all reaction does not involve net oxidation.

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N-FORMIMINO-L-ASPARTIC ACID AS AN INTERMEDIATE IN THE ENZYMATIC CONVERSION OF IMIDAZOLE- ACETIC ACID TO FORMYLASPARTIC ACID*

By OSAMU HAYAISHI,† HERBERT TABOR, AND TAKIKO HAYAISHI

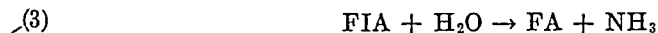
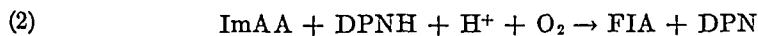
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In earlier communications (1, 2) we have described the conversion of imidazoleacetic acid¹ to formylaspartic acid by a partially purified enzyme preparation obtained from a pseudomonad grown on ImAA. This reaction required DPNH and oxygen, as shown in Reaction 1



The present communication is concerned with the further purification of the enzyme system and with the identification of *N*-formimino-*L*-aspartic acid as an intermediate compound in Reaction 1. Separate enzymes, ImAA oxidase and FIA hydrolase, which have been purified, carry out Reactions 2 and 3, respectively



Methods

Materials—ImAA was prepared in collaboration with Dr. Hugo Bauer by the alkaline hydrolysis of imidazoleacetoneitrile; the latter was obtained by the treatment of histidine with sodium hypochlorite (3). C¹⁴-Carboxyl-labeled ImAA was obtained by the same method by starting with commercial α -C¹⁴-DL-histidine.

FIA was synthesized by a modification of the formiminoalanine prepa-

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¹ The following abbreviations are used throughout the paper: ImAA, β -(imidazolyl-4(5))-acetic acid, FIA, *N*-formiminoaspartic acid (formamidosuccinic acid), FA, formylaspartic acid, DPN, diphosphopyridine nucleotide, TPN, triphosphopyridine nucleotide, DPNH, reduced DPN, TPNH, reduced TPN, Tris, tris-(hydroxymethyl)aminomethane, EDTA, ethylenediaminetetraacetic acid, PCMB, *p*-chloromercuribenzoate.

ration of Micheel and Flitsch (4), which involves the condensation of formamidine and aspartic acid in formamide. Formamidine hydrochloride was prepared by the reductive desulfurization of thiourea with Raney nickel according to Brown (5). To prepare FIA, 1.6 gm (20 mmoles) of formamidine hydrochloride, 3 gm (11 mmoles) of Ag_2CO_3 , 1.33 gm (10 mmoles) of L-aspartic acid, and 5 ml of redistilled formamide were vigorously stirred for 36 hours at room temperature in a 50 ml round-bottomed flask, stoppered with a CaCl_2 tube. The mixture was then placed *in vacuo* for $\frac{1}{2}$ hour to remove most of the dissolved CO_2 , treated with 100 ml of 0.06 N HCl, and filtered. The FIA in the filtrate was adsorbed on a Dowex 1 acetate column (8 to 10 per cent cross-linked, 200 to 400 mesh, 4.5 sq cm \times 18 cm) and eluted by gradient elution (0.3 N acetic acid was added to a mixing reservoir containing 250 ml of H_2O), it appeared in the 250 to 450 ml fraction, and was detected by the alkaline ferricyanide-nitroprusside color test of Rabinowitz and Pincer (6). Free aspartic acid was eluted in the succeeding fractions and was most easily detected by paper chromatography of aliquots (see below). After evaporation of the FIA fractions *in vacuo*, 1.1 gm (69 per cent yield, based on aspartic acid) of crystalline FIA were obtained. This material was then recrystallized from warm 50 per cent ethanol, m p 190–198° (with decomposition).

$\text{C}_6\text{H}_8\text{N}_2\text{O}_4$	Calculated	C 37.50, H 5.05, N 17.50
	Found	" 37.45, " 4.93, " 17.28

Titration of 0.05 to 0.1 M solutions indicated that pK_1' , pK_2' , and pK_3' were approximately 2.2, 4.1, and 11.6. FIA is stable for long periods (at least several months) at room temperature in 6 N HCl, 0.1 N HCl, 0.1 N acetic acid, 0.1 M acetate buffer, pH 4, and 0.2 M potassium phosphate buffer, pH 7.2. It has a half life at room temperature of approximately 40 hours in 0.05 M sodium borate, pH 9.2, 70 minutes at pH 10.8, 20 minutes at pH 11.5, and 10 minutes in 1 N KOH. After hydrolysis in 0.1 N NaOH, no FIA could be detected, and 1 equivalent each of L-aspartic acid, formic acid, and ammonia was produced. In *tert*-butanol 70, formic acid 15, H_2O 15 (7) solvent with Whatman No. 1 paper, FIA moved with an R_F of 0.48, a small impurity of aspartic acid (0.1 per cent) could be detected with an R_F of 0.32. FA was synthesized according to Tabor and Mehler (8). Formiminoglycine was kindly furnished by Dr. J. C. Rabinowitz. 5-Carboxymethylhydantoin was a gift from Dr. I. Lieberman.

Glucose dehydrogenase was purified from beef liver (9) and contained 3.8 mg of protein (3020 units) per ml. Crystalline catalase was obtained from the Worthington Biochemical Corporation. DPN, TPN, DPNH, TPNH, and glucose oxidase (notatin) were obtained from the Sigma Chemical Company.

Determinations—Protein was determined by the phenol method (10), aspartic acid was measured with aspartic decarboxylase (11), and formate was measured by a manometric procedure (12). Ammonia was determined by an unpublished method of S M Rosenthal. A sample containing 0.2 to 1 μ mole of ammonia was placed on an XE-64 K^+ column (2 cm \times 1 sq cm), and the column was washed twice with about 4 ml of water with pressure. This procedure washed out all the FIA, which reacts slowly with the alkaline Nessler's reagent. The column was then eluted with 2.5 ml of 0.2 N NaOH, followed by 0.5 ml of water. The amount of ammonia in the eluate was then determined by direct nesslerization with 0.2 ml of an alkali-free Nessler's reagent.

Whatman No. 3 MM paper was used for paper chromatography, which was carried out by a descending technique at room temperature with the solvent systems described in the text. Aspartic acid was detected on the chromatograms by a ninhydrin spray and heating at 90° for 5 minutes. FIA and FA were rendered visible with ninhydrin after exposure of the paper to NH_3 and HCl vapor, respectively, for several hours. FIA was also rendered visible by the alkaline ferricyanide-nitroprusside reagent (6) and FA by the chlorine method (13). Ion exchange chromatography was carried out on Dowex 50 (2 per cent cross-linked, 200 to 400 mesh, hydrogen form) and Dowex 1 (2 per cent cross-linked, 200 to 400 mesh, acetate form) columns. C^{14} -containing samples were plated as thin layers on metal disks and measured in a gas flow counter.

Enzyme Assay

ImAA Oxidase—The standard assay system contained the following components, expressed in micromoles, in a total volume of 1 ml: Tris buffer, pH 9.0, 10; ImAA 5; DPNH 0.2; and enzyme. The reaction was started by the addition of enzyme, and the decrease of absorption at 340 $m\mu$ was measured at 30 second intervals over 5 minutes at 25° in a Beckman model DU spectrophotometer with cells having 1 cm light path. A unit of enzyme was defined as the amount oxidizing 1 μ mole of DPNH during a 1 hour period under these conditions, and the specific activity was defined as units per mg of protein. Correction for DPNH oxidase activity as measured in controls without ImAA was made when crude enzyme preparations were employed.

FIA Hydrolase—FIA was determined according to the method described by Rabinowitz and Pricer for the determination of formiminoglycine (6). This standard assay system for the enzyme activity contained, in 0.5 ml, 4 μ moles of FIA, 50 μ moles of Tris buffer, pH 9.0, and enzyme protein. Incubation was carried out in 3 ml Beckman cuvettes with a 1 cm light path at 25°, and, after 15 minutes, 2.0 ml of saturated sodium borate

solution were added to stop the reaction. This was followed by the addition of 0.5 ml of the alkaline ferricyanide-nitroprusside reagent, and the optical density was determined at 500 m μ after 30 minutes at 25°. 1 μ mole of FIA gave an optical density of approximately 0.500. A unit of enzyme is defined as the amount hydrolyzing 1 μ mole of FIA in 1 hour, calculated from the disappearance of FIA during 15 minutes. The specific activity is defined as units of enzyme per mg of protein.

The intensity of the color obtained with FIA in this procedure is variable, depending on the concentration and kind of salts present. For example, 50 μ moles of Tris buffer, under the standard assay conditions, gave no color development by itself, but intensified the color development with FIA by approximately 20 per cent.

Growth Conditions and Preparation of Crude Extracts—*Pseudomonas* sp (ATCC 11299B)² was grown with shaking in a 6 liter Erlenmeyer flask containing 1 liter of medium. The medium contained 1.5 gm of K₂HPO₄, 0.5 gm of KH₂PO₄, 0.2 gm of MgSO₄ · 7H₂O, 1.0 gm of ImAA hydrochloride, and 1.0 gm of Difco yeast extract per liter of distilled water. The pH was adjusted to 7.0 with 1 N KOH.

Growth was allowed to proceed at 25° for 18 hours, and the cells were then harvested by centrifugation and washed. The yield of cells was approximately 2.0 gm of wet weight per liter of medium. Cell-free extracts were prepared by grinding the cells with alumina (Alcoa A-301) (14) and extracting with 20 volumes of 0.02 M potassium phosphate buffer, pH 7.0. Crude extracts usually contained approximately 3.4 mg of protein per ml, and the specific activity of ImAA oxidase varied between 5 and 10.

Results

ImAA Oxidase

Enzyme Purification—To 100 ml of the crude extracts were added 50 ml of a 1 per cent solution of protamine sulfate (Eli Lilly and Company), and the precipitate was removed by centrifugation (protamine supernatant fraction).

To 100 ml of the protamine supernatant fraction was added sufficient calcium phosphate gel (15) to adsorb 90 to 100 per cent of the enzyme activity. Usually 10 ml (165 mg, dry weight) were required. After 10 minutes, the mixture was centrifuged and the supernatant fluid was discarded. The gel was washed once with 20 ml of 0.04 M potassium phosphate buffer, pH 7.0, and then the enzyme was eluted from the pre-

² *Pseudomonas* sp ATCC 11299 contains two colony types. A large, flat and smooth colony, A, and a small and elevated type, B, were distinguished only by the colony appearance. No difference has been detected between the two colony types in their enzymatic or biochemical behavior.

precipitate with two batches of 20 ml each of 0.2 M potassium phosphate buffer, pH 7.0 (calcium phosphate gel fraction I)

The eluates were combined and passed through a Dowex 1 formate column (1 sq cm \times 12 cm) at 4° in order to replace phosphate ions by formate ions. Sufficient aluminum hydroxide gel C γ (16) (usually about 14 ml, containing 260 mg of solids per 70 ml of the protein solution) was added to adsorb most of the enzyme activity. The precipitate was collected after 10 minutes and washed once with 20 ml of 0.005 M potassium phosphate buffer, pH 7.0. The enzyme was then eluted successively with 20 ml of 0.01 M potassium phosphate buffer, pH 7.0, and two portions of 20 ml of 0.02 M potassium phosphate buffer, pH 7.0 (aluminum hydroxide gel fraction I).

The three fractions were combined and passed through a short Dowex 1 formate column (2 cm \times 1 sq cm) at 4°. Sufficient calcium phosphate gel (usually 6 ml per 60 ml of the protein solution) was added to adsorb most of the activity. The gel was washed with 12 ml of 0.02 M potassium phosphate buffer, pH 7.0, then eluted successively with two portions of 12 ml each of 0.1 M potassium phosphate buffer, pH 7.0 (calcium phosphate gel fraction II).

The combined eluates were passed through a Dowex 1 formate column (5 cm \times 1 sq cm) and to the enzyme solution (25 ml) were added 2.5 ml of the aluminum hydroxide gel suspension. After 10 minutes the gel was centrifuged, washed once with 10 ml of 0.005 M potassium phosphate buffer, pH 7.0, and then the enzyme was eluted twice with 10 ml each of 0.01 M potassium phosphate buffer, pH 7.0 (aluminum hydroxide gel fraction II).

The combined eluates were passed through Dowex 1 formate column (2 cm \times 1 sq cm), and then the calcium phosphate gel treatment was carried out as before. The enzyme was eluted successively with 5 ml portions of 0.02 M and 0.04 M potassium phosphate buffer, pH 7.0. A typical result is shown in Table I. Approximately 220-fold purification was achieved with about 18 per cent yield. Calcium phosphate gel fraction III was free from detectable DPNH oxidase activity but still contained a trace of FIA hydrolase.

Isolation and Identification of FIA—Accumulation of an unidentified compound during the oxidation of ImAA by a partly purified enzyme preparation was noted previously (2). With more purified enzyme preparations it was possible to accumulate this compound in sufficient amounts for isolation. The reaction mixture (10 ml) contained 1000 μ moles of Tris buffer, pH 8.9, 1000 μ moles of D-glucose, 3 μ moles of DPN, 25 μ moles of carboxyl-labeled ImAA (95,000 c.p.m.), 0.2 ml of glucose dehydrogenase, and about 20 units of aluminum hydroxide gel fraction I. Incu-

bation was carried out in a 150 ml Erlenmeyer flask which was shaken gently on a reciprocal shaker. After 45 minutes at 25°, the reaction was stopped by the addition of 0.5 ml of 70 per cent perchloric acid, and the precipitate was removed by centrifugation. The supernatant solution was then placed on a Dowex 50 hydrogen form column (10 cm × 1 sq cm),

TABLE I
Purification of ImAA Enzyme

Enzyme fraction	Total units	Over all recovery, per cent	Specific activity
Crude extract	1280		6.6
Protamine supernatant	1200	94	8.0
Calcium phosphate gel I	1140	89	63.0
Aluminum hydroxide gel I	860	67	167.0
Calcium phosphate " II	745	58	201.0
Aluminum hydroxide " II	485	38	785.0
Calcium phosphate " III	230	18	1423.0

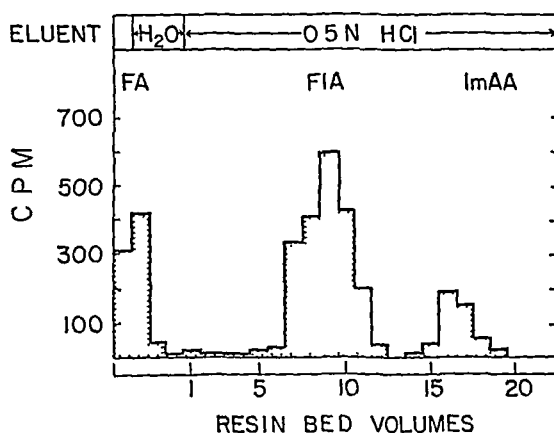


FIG 1 Ion exchange chromatogram. Conditions as described in the text. The total recovery of the radioactivity was 81.8 per cent.

washed with water, and eluted with 0.5 N HCl. As shown in Fig 1, approximately 15 per cent of the radioactivity came through the column when it was washed with water. This fraction contained FA, as previously reported (1, 2).

The second peak appeared between 7 and 11 resin bed volumes, these fractions were pooled and condensed *in vacuo* at 25° with a flash evaporator. This fraction usually represented about 40 to 60 per cent of the total radioactivity. The enzymatic synthesis of larger amounts of FIA proved difficult, due mainly to the rapid decrease in the reaction rate when the more

purified preparations were used. The last peak, which came out after 15 resin bed volumes, contained the remaining ImAA.

Samples of the second peak collected from ten flasks were pooled and dissolved in about 10 ml of cold water, and the pH was cautiously adjusted to about 6.0. This was chromatographed on a column of Dowex 1, acetate form (5 cm \times 1 sq cm), and eluted with 2 N acetic acid. The radioactive fractions which appeared as a single peak between 8 and 16 resin

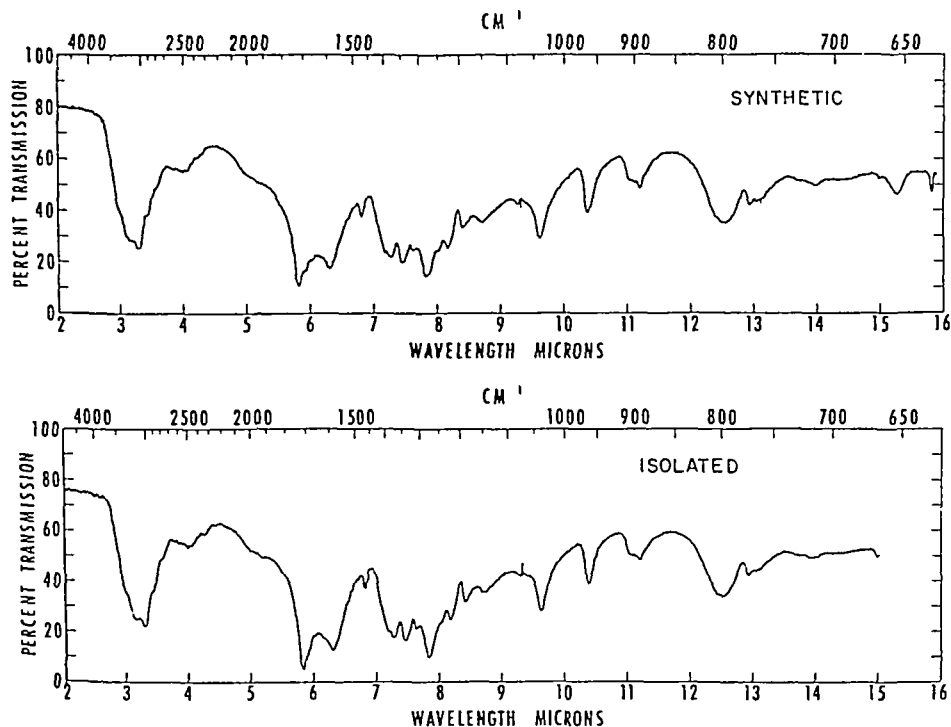


FIG. 2. Infrared absorption spectra of synthetic and isolated FIA were made with suspensions in KBr with a Perkin-Elmer recording spectrophotometer. We are indebted to Mr. H. K. Miller for these measurements.

bed volumes were combined, and water and acetic acid were removed by lyophilization. The white semicrystalline material weighed 11 mg (yield, 27.5 per cent). This material was dissolved in 0.5 ml of water, and about 2.0 ml of absolute alcohol were added. Upon standing at 4° overnight, 6 mg of crystals were obtained.

The identity of this material with the synthetic FIA was established by the following criteria: (1) elementary analysis: $C_5H_8N_2O_4$. Calculated, C 37.50, H 5.01, N 17.50, found, C 37.45, H 5.05, N 17.14. (2) Infrared spectra, as shown in Fig. 2. (3) R_F values, two different solvent systems being used (Table II). (4) Both synthetic and isolated products started to darken at about 162° when heated slowly on a Kofler block, and melted

at 190–198° with decomposition (5) When treated with the sodium borate and ferricyanide-nitroprusside reagent, the time-course of the development and the spectrum of the color were identical (Fig 3)

TABLE II
R_F Values of FIA, FA, and Aspartic Acid

	Solvent system	
	<i>tert</i> Butanol formic acid water (70 15 15)	77 per cent ethanol
FIA	0.47 ~ 0.51	0.42 ~ 0.43
FA	0.65 ~ 0.66	0.51 ~ 0.53
Aspartic acid	0.31 ~ 0.34	0.27 ~ 0.29
Isolated FIA	0.48	0.42
“ “ after 0.1 N NaOH, 100°, 5 min	0.33	0.27
“ “ “ hydrolysis by FIA hydrolase	0.65	0.53

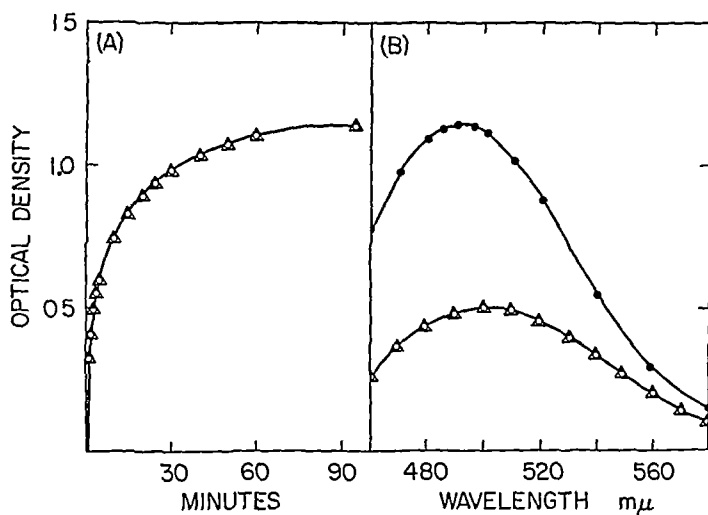


FIG 3 Time-course of color development and spectra of FIA when treated with alkaline ferricyanide-nitroprusside reagent. Conditions as described in the text. A, optical density increase was determined at 500 mμ. The amount of FIA was 2 μmoles, O, synthetic compound of FIA, Δ, isolated material. B, O, authentic compound of FIA, Δ, isolated material, ●, formiminoglycine. 1 μmole was used in each case.

Alkaline hydrolysis (0.1 N NaOH, 100°, 5 minutes) resulted in the formation of free aspartic acid which was determined quantitatively by an enzymatic method (11). Since the enzyme reacts only with the L form, both the hydrolyzed aspartic acid and the original FIA had the L configuration.

Specificity—TPNH does not replace DPNH. Imidazole, imidazole-lactic acid, histidine, succinic acid, histamine, and 5-carboxymethylhydantoin were all completely inactive as substrates. Imidazolepropionic acid was about 3 per cent as active as ImAA, presumably yielding formiminoglutamic acid as a product.

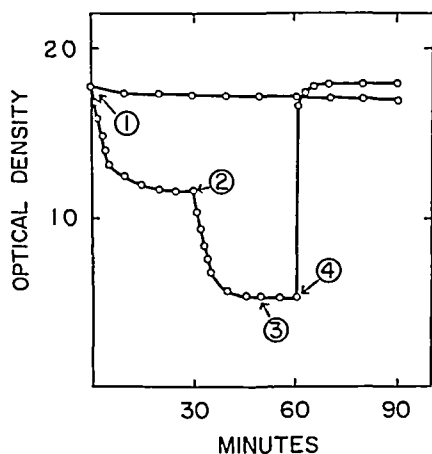


FIG 4

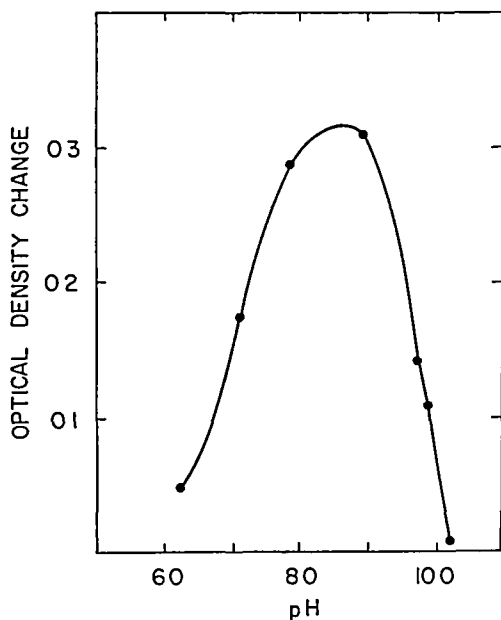


FIG 5

FIG 4 Stoichiometry and irreversibility. The reaction mixture (10 ml) contained 100 μ moles of Tris buffer, pH 9.0, 0.3 μ mole of DPNH, and 0.9 unit of enzyme (aluminum hydroxide gel fraction I). 0.1 μ mole of ImAA was added at zero time (indicated by arrow 1) and at arrow 2. At arrow 3, 0.5 μ mole of FIA was added. At arrow 4, 50 μ moles of glucose and glucose dehydrogenase (20 units) were added.

FIG 5 Rate of reaction as a function of pH. Standard assay conditions, except that phosphate buffer was used for pH 6.25, Tris buffer for pH 7.1, 7.9, 8.9, and 9.75, and glycine buffer for pH 9.9 and 10.2.

Reversibility—When FIA was incubated with the enzyme and DPN, no reduction of DPN occurred either aerobically or anaerobically. Similarly, when radioactive FIA was incubated with ImAA, DPNH, and enzyme, no radioactivity was found in the remaining ImAA. Reaction 2 was therefore practically irreversible. These observations are consistent with the stoichiometric relation between the oxidation of DPNH and the amount of ImAA added in the standard assay conditions (Fig 4). This enzyme accordingly provides a convenient tool for the specific quantitative determination of ImAA.

Effect of pH and Substrate Concentration—Under the standard assay

conditions, maximal rates were obtained in the pH range 8.0 to 9.0 (Fig. 5). The rate of DPNH oxidation in the presence of ImAA and the en-

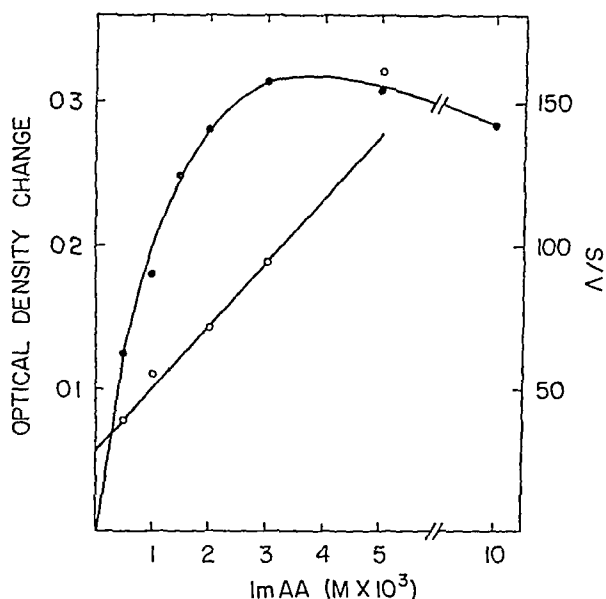


FIG. 6 Rate of reaction as a function of substrate concentration (●). The right hand ordinate scale refers to a Lineweaver-Burk plot (○) of substrate concentration divided by velocity (s/v)

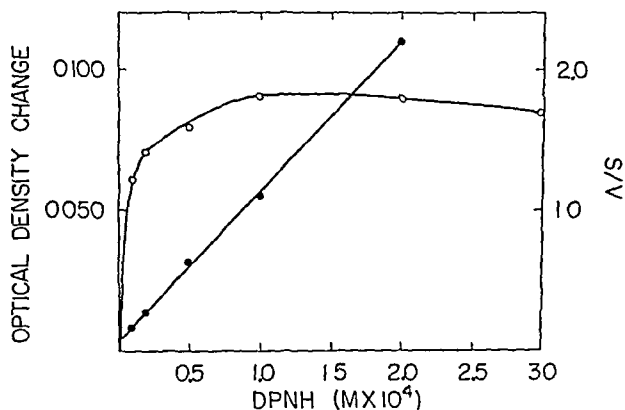


FIG. 7 Rate of reaction as a function of DPNH concentration (○). The right hand ordinate scale refers to a Lineweaver-Burk plot (●) of substrate concentration divided by velocity (s/v)

zyme as a function of ImAA and DPNH concentration is shown in Figs. 6 and 7. The K_s values were found to be approximately 1.3×10^{-3} M for ImAA and about 0.8×10^{-5} M for DPNH under standard assay conditions.

Effect of Inhibitors—In order to study the nature of the enzyme, the effect of several inhibitors was investigated (Table III)

As *p*-chloromercuribenzoate inhibited the enzyme activity, the sulfhydryl group is apparently essential for the activity. Metal ions (probably copper ions) appear to be involved, since 8-hydroxyquinoline sulfonate and sodium diethyldithiocarbamate exerted marked inhibition. On the

TABLE III
Inhibition Experiment

Standard assay system The optical density change was determined for 2 minutes

Inhibitors	Concentration, M	Per cent inhibition	Inhibitors	Concentration, M	Per cent inhibition
Sodium diethyldithiocarbamate	1×10^{-3}	28	Chel 330*	5×10^{-3}	0
Sodium diethyldithiocarbamate	2×10^{-3}	42	“ 600†	5×10^{-3}	0
Sodium diethyldithiocarbamate	5×10^{-3}	64	Sodium oxalate	10^{-2}	0
Diphenylthiocarbazonc	2×10^{-3}	0	KCN	5×10^{-2}	0
EDTA	10^{-2}	0	Sodium azide	5×10^{-2}	0
8 Hydroxyquinoline sulfonate	1×10^{-3}	43	“ fluoride	5×10^{-2}	0
8 Hydroxyquinoline sulfonate	2×10^{-3}	100	PCMB	10^{-6}	51
O Phenanthroline	5×10^{-3}	18	“	10^{-4}	100
α, α -Dipyridyl	5×10^{-3}	0	Catalase	2.5 units per ml	+20

* Chel 330, cyclohexane-1,2-diaminetetraacetic acid

† Chel 600, diethylenetriaminepentaacetic acid. Samples of Chel 330 and 600 were generously supplied by Mr. H. Kroll of the Geigy Chemical Corporation, Providence, Rhode Island.

other hand, other chelating reagents, such as EDTA, KCN, etc., did not show any inhibition.

FIA Hydrolyase

Adaptive Behavior of Pseudomonas Cells—The organism, which was grown in the presence of ImAA, could oxidize ImAA as well as FA and aspartic acid without a lag period, but non-adapted cells showed a considerable adaptive lag before oxidation took place (2). Both of these compounds were therefore implicated as intermediates of ImAA metabolism. However, when oxygen uptake was determined by a suspension of ImAA-adapted cells with FIA as a substrate, the rate of oxidation was remarkably low and there was little difference between ImAA-adapted cells and

cells grown with glucose and ammonium sulfate (Table IV) This low activity towards FIA, however, appears to be due to cell permeability, since, when the cells were broken by grinding with alumina and the extracts were tested for FIA-degrading activity, extracts from non-adapted cells were practically inactive, whereas the specific activity of FIA hydrolase from ImAA-adapted cells was even higher than that of ImAA oxidase under the conditions employed

TABLE IV
Adaptive Nature of Organism

Experiment 1, each Warburg vessel contained, in 20 ml, 4 μ moles of substrate, 100 μ moles of potassium phosphate buffer, pH 7.0, and about 10 mg of cell material. The numbers represent the oxygen consumption during the first 30 minutes, corrected for endogenous oxidation expressed as microliters. Experiment 2, extracts were assayed with the standard assay method. The numbers represent specific activities.

Substrate	Cell suspension, Experiment 1		Extracts, Experiment 2	
	ImAA adapted	Non adapted	ImAA adapted	Non adapted
ImAA	189	3	7.0	0.0
FIA	6	1	31.0	0.0

TABLE V
Purification of FIA Hydrolase

Fraction No		Total units	Specific activity
1	Crude extract	3830	31
2	Protamine supernatant	3720	40
3	Aluminum hydroxide gel eluate	1420	276
4	Calcium phosphate " "	435	615

Purification of FIA Hydrolase—All operations were carried out at 0–3°. Crude extracts were prepared as described above.

To 50 ml of crude extract were added, with stirring, 10 ml of a 1 per cent protamine solution (Eli Lilly and Company). After removal of the precipitate by centrifugation, cold water was added to the supernatant solution to make a total volume of 100 ml.

The protamine supernatant fraction (100 ml) was treated with 10 ml of aluminum hydroxide gel C γ (18.3 mg of solids per ml). After 15 minutes at 0°, the gel was collected by centrifugation and washed with 20 ml of 0.04 M potassium phosphate buffer, pH 7.0, and then the enzyme was eluted with 20 ml of 0.1 M potassium phosphate buffer, pH 7.2 (aluminum hydroxide gel eluate, Table V).

The aluminum hydroxide gel eluate (20 ml) was diluted to 200 ml with water, and then 10 ml of calcium phosphate gel (16.5 mg of solids per ml) were added slowly with stirring. After 15 minutes at 0°, the gel was washed with 10 ml of 0.02 M potassium phosphate buffer, pH 7.0, and then the enzyme was eluted with 10 ml of 0.05 M potassium phosphate buffer, pH 7.0 (calcium phosphate gel eluate).

The aluminum hydroxide gel eluate retained almost all the activity after being kept at -15° for a month.

Isolation and Identification of FA—In order to obtain a sufficient amount of the product of FIA breakdown for positive identification, a large scale reaction was carried out with an incubation mixture containing 64 mg (400 μ moles) of FIA, 2 mmoles of Tris buffer, pH 9.0, and aluminum hydroxide gel eluate (900 units) in a total volume of 20 ml. At intervals during incubation at 25°, aliquots of the reaction mixture were taken out and, after proper dilution, assayed for FIA disappearance. When FIA removal was complete (60 minutes), the incubation mixture was chilled to 0°, the pH was adjusted to 3.0 with N HCl, and the precipitated protein was discarded after centrifugation. The supernatant solution was then passed through a Dowex 50 (H⁺ form, 1 sq cm \times 10 cm) column.

The Dowex-treated solution was neutralized carefully and was subjected to ion exchange chromatography on a column of Dowex 1 acetate (1 sq cm \times 10 cm), and elution was carried out with 2 N acetic acid. Aliquots (0.1 ml) were taken out from each fraction, mixed with 0.1 ml of 2 N HCl, hydrolyzed at 100° for 10 minutes, and then assayed by the reduced ninhydrin procedure of Moore and Stein (17) as employed by Tabor and Mehler for the isolation and identification of formylglutamic acid (8).

The acid-labile ninhydrin-positive fraction was eluted from the column between 8 and 15 resin bed volumes of eluent, with a peak at 11. The combined fraction was lyophilized and the residue was extracted with about 10 ml of ethanol. Insoluble material was removed by centrifugation, and the supernatant fluid was condensed to about 2 ml under reduced pressure at a water bath temperature of 40–50°. Several volumes of benzene were added, and about 40 mg of semicrystalline material, m.p. 132°, were obtained. Recrystallization from hot acetone and benzene yielded 23 mg of crystalline material.

The enzymatic product was identified as FA on the basis of the following criteria: (1) identity of its infrared spectrum with that of the authentic compound (Fig. 8), (2) elementary analysis: C₅H₇O₅N. Calculated, C 37.27, H 4.37, N 8.70, found, C 37.44, H 4.34, N 8.54, (3) melting point of isolated FA, 135–137°, that of the authentic FA, 135–137°, mixed m.p., 135–137°, (4) identical chromatographic behavior on Dowex 1 and on paper.

Stoichiometry—Balance studies were made with C¹⁴-labeled FIA as a

substrate The β -carboxyl-labeled FIA was prepared enzymatically from C^{14} -carboxyl-labeled ImAA The incubation mixture (0.8 ml), containing 6 μ moles of FIA (2000 c.p.m.), 25 μ moles of Tris buffer, pH 9.0, and 18 units of enzyme (aluminum hydroxide gel fraction), was incubated at 25°. At zero time and after 10 and 20 minutes incubation, aliquots were removed and the amount of remaining FIA and ammonia was determined as described under "Methods" When the deproteinized reaction mixture

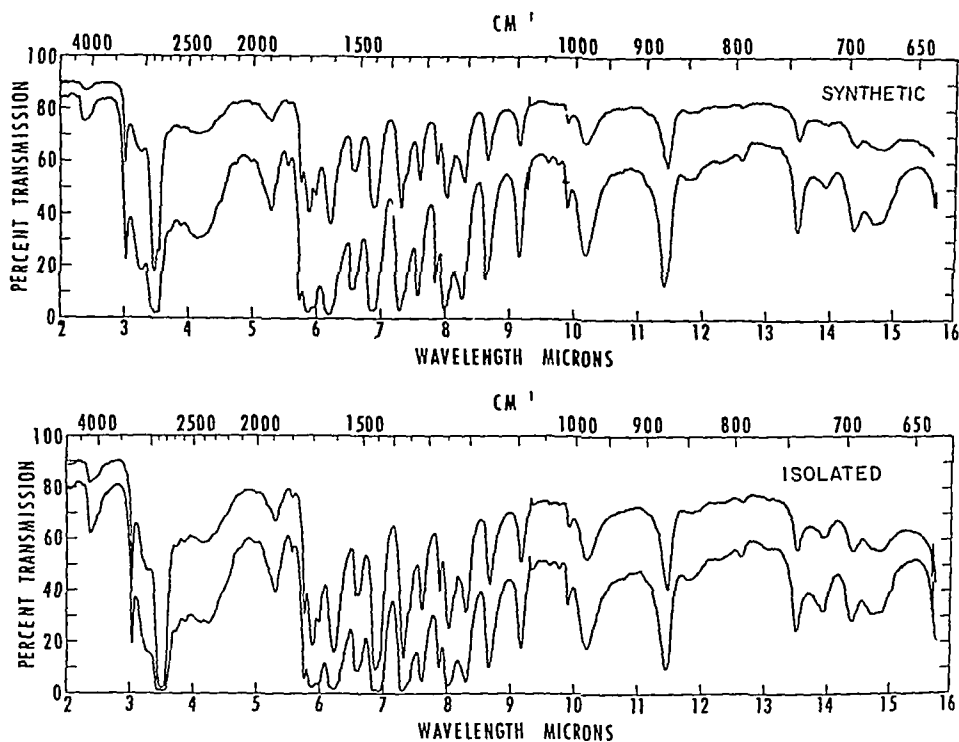


FIG 8 Infrared absorption spectra of synthetic and isolated FA were made with suspensions in Nujol with a Perkin-Elmer recording spectrophotometer We are indebted to Mr W Jones for the determination

was chromatographed on paper, only one spot appeared as a product of the reaction, which corresponded to FA in R_f values (Table II) The spot corresponding to FA was cut out, eluted with water, and radioactivity was counted As shown in Table VI, the results indicate that the over-all reaction was the stoichiometric hydrolysis of FIA to yield FA and ammonia

Mechanism of Reaction—The following experiment demonstrated that the FA produced from FIA was not formed by a secondary reaction involving aspartic acid and formic acid FIA degradation was carried out in the presence of radioactive aspartic acid and, when more than 90 per cent of FIA was converted to FA, the reaction mixture was deproteinized and chromatographed on paper As shown in Fig 9, radioactivity in the

aspartic acid was not incorporated into FA at all, indicating that free aspartic acid is not an intermediate in the enzymatic formation of FA from FIA

TABLE VI
Balance Studies of FIA Hydrolase

The conditions are as described in the text

Incubation	FIA	NH ₃	FA
min	μmole	μmoles	μmoles
10	-2.76	+2.79	+2.35
20	-5.03	+4.95	+4.92

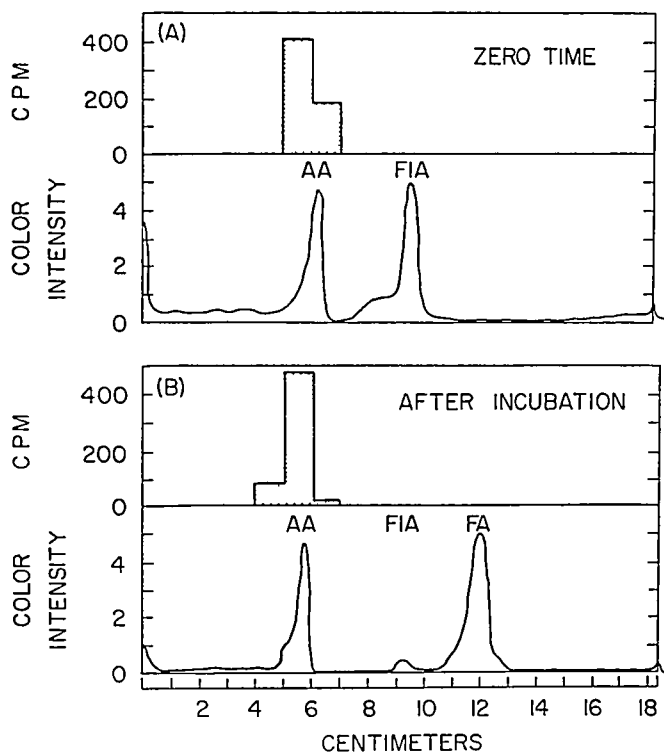


FIG. 9. FIA hydrolase reaction in the presence of labeled aspartic acid. Incubation mixture (0.6 ml) contained 8 μmoles of FIA, 100 μmoles of Tris buffer, pH 9.0, 0.8 μmole of L-aspartic acid (24,000 c.p.m.), and 0.1 ml of aluminum hydroxide gel fraction. At zero time (A) and after 30 minutes incubation at 26° (B), 0.3 ml aliquots were removed and deproteinized with 5 per cent perchloric acid. To the supernatant fluid was added 1 μmole of L-aspartic acid as a carrier, and perchloric acid was neutralized with KOH. Aliquots (0.05 ml) were applied to Whatman No. 3 MM paper, and the chromatograms were developed by descending technique with a solvent containing 70 parts of *tert*-butanol, 15 parts of formic acid, and 15 parts water (7) for 8 hours. The spots were rendered visible and counted as described under "Methods."

Preliminary experiments have not yet produced any evidence for a folic acid type of cofactor in this degradation. This, therefore, appears to be different from the reported involvement of tetrahydrofolic acid in the degradation of formiminoglycine (18, 19) and formiminoglutaric acid (20, 21) by *Clostridium cylindrosporum* and rabbit liver, respectively. No stimulation of FIA hydrolase was observed in crude extracts of *Pseudomonas* or in the partially purified FIA hydrolase (with and without Dowex 1 formate treatment) upon addition of *C. cylindrosporum* extracts³ or heat-inactivated crude extracts of *Pseudomonas* (80°, 5 minutes).

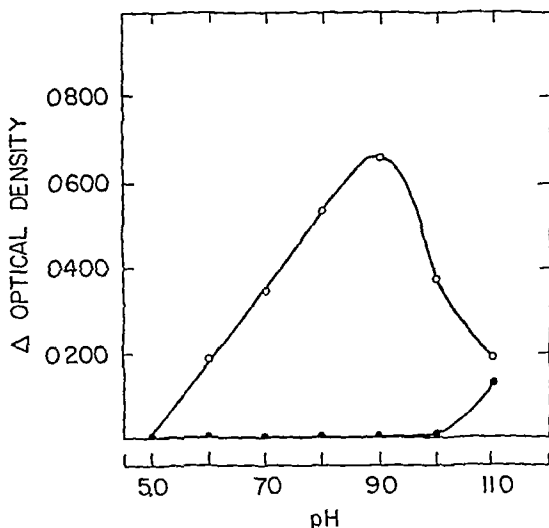


FIG 10 Rate of reaction as a function of pH. Standard assay conditions, except that the following buffers were used: acetate, pH 5.0; phosphate, pH 6.0, 7.0; Tris, pH 8.0, 9.0; carbonate, pH 10.0, 11.0. ●, spontaneous decomposition; ○, enzymatic degradation.

Specificity—FIA hydrolase appears to be a specific enzyme for FIA, since neither formiminoglutaric acid nor formiminoglycine was hydrolyzed at an appreciable rate. The enzymatic hydrolysis of FIA was not inhibited by the presence of equimolar concentrations of either formiminoglutaric acid or formiminoglycine.

Effect of pH and Substrate Concentration—Under the standard assay conditions, maximal rates were obtained at pH 9.0 (Fig 10). Above pH 10.0 FIA was hydrolyzed spontaneously at a slow rate. About 8 per cent was decomposed in 15 minutes at 25°, pH 11.0.

The rate of enzymatic hydrolysis as a function of substrate concentration is shown in Fig 11. The K_s value as determined by the Lineweaver-

³ Kindly furnished by Dr. J. C. Rabinowitz.

Burk plot (22) was found to be approximately 2.6×10^{-3} M under these conditions

Effect of Inhibitors—Under standard assay conditions, the following compounds did not show any inhibition KCN (0.04 M), KF (0.1 M), and EDTA (0.025 M). *p*-Chloromercuribenzoate (0.00025 M) showed 25 per cent inhibition. When the enzyme was incubated with 0.05 M EDTA overnight at 0° and then tested for activity, no inhibition was observed as compared to the enzyme treated in the same way but without EDTA.

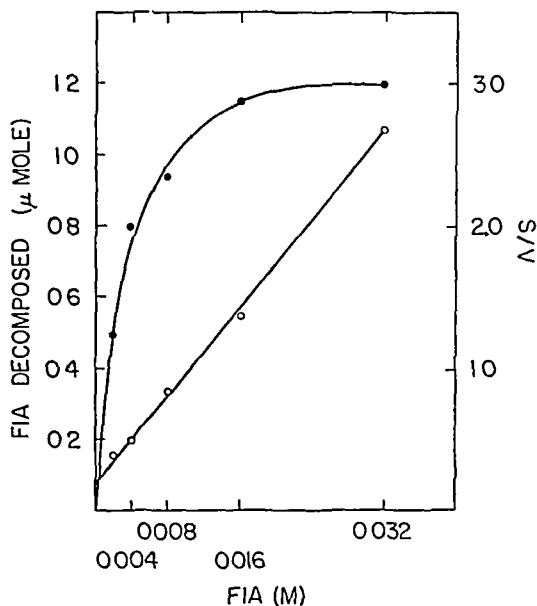


Fig. 11 Rate of reaction as a function of substrate concentration (●) Standard assay conditions (○) Lineweaver-Burk plot of s/v versus substrate concentration

DISCUSSION

The evidence presented in this paper establishes the identity of the enzymatically produced compound in the IMAA degradation by *Pseudomonas* enzyme with synthetic FIA. The natural occurrence of foimimino derivatives of glutamic acid and glycine and their metabolism has recently been reported from several laboratories. Foimiminoglutamic acid (8, 23, 24) has been synthesized and found to be an intermediate in the enzymatic degradation of histidine by mammalian liver preparations and by histidine-adapted *Pseudomonas* extracts. The conversion of FIA to EA and ammonia reported here appears similar to the degradation of foimiminoglutamic acid to formylglutamic acid and ammonia in histidine-adapted *Pseudomonas* preparations (8). Other reported pathways for the metabolism of

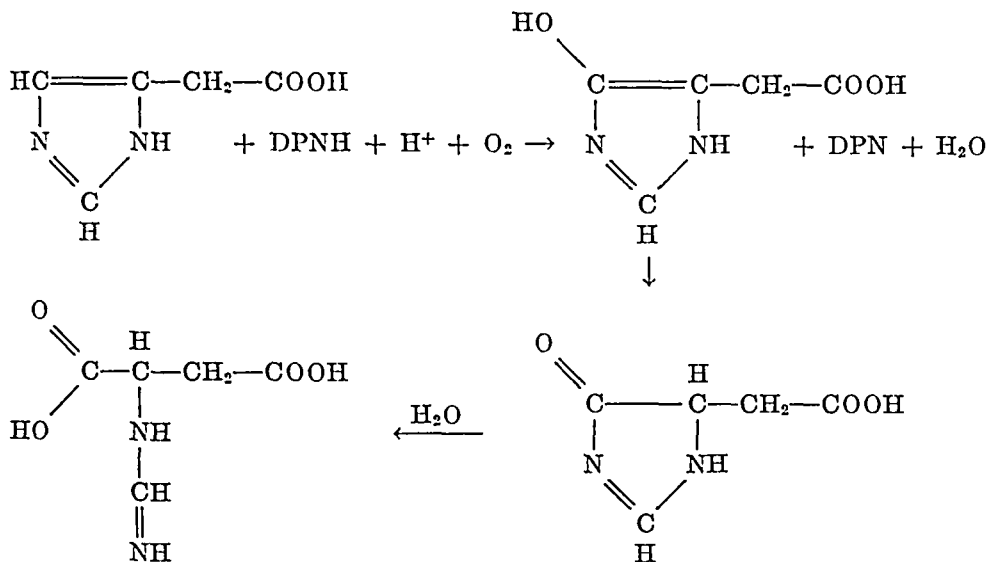
formimino compounds are the conversion of formiminoglutamic acid to formamide and glutamic acid in extracts of *Aerobacter aerogenes* (25) and *Clostridium tetanomorphum* (26) and the conversion of formiminoglycine to glycine, formate, and ammonia by a partially purified enzyme preparation from *C. cylindrosporum* (6). More recently there has been recorded the conversion of formiminoglycine and formiminoglutamic acid to 5-formiminotetrahydrofolic acid and the corresponding amino acids in extracts of *C. tetanomorphum* (27) and rabbit liver (21), respectively.

The nature of ImAA oxidase as well as the mechanism of the reaction remains unclarified. As previously reported (1, 2), disappearance of ImAA from the incubation mixture and the oxidation of DPNH in the presence of ImAA are dependent on the presence of both DPNH and oxygen. When the complete system was incubated first aerobically without DPNH and then anaerobically in the presence of DPNH, no oxidation of DPNH was observed, the reaction took place immediately after air was introduced to the reaction vessels. Anaerobic incubation of the complete system did not yield any evidence for the disappearance of ImAA as determined by a diazo reaction (3) or by ion exchange chromatography. Throughout extensive purification of the enzyme, no evidence was obtained for the presence of more than one protein fraction for this activity.

Simultaneous requirement for reduced pyridine nucleotides and oxygen for certain aerobic reactions has been observed in other instances. Thus, in the case of phenylalanine hydroxylase, both DPNH and oxygen were required for the conversion of phenylalanine to tyrosine (28). TPNH and oxygen were both required for oxidative reactions with various drugs (29, 30), as well as hydroxylation of steroids (31) and kynurenine (32, 33). It is possible that reduced pyridine nucleotides are oxidized to generate H_2O_2 or some form of peroxide on the enzyme surface, which is then utilized for the oxidative reactions. In fact, photolysis of ImAA by ultraviolet light was shown to produce aspartic acid and formic acid by non-enzymatic reactions in which H_2O_2 seems to be involved (34). However, this interpretation is rendered less likely, since there is no measurable DPNH oxidase in the purified preparation and glucose and notatin could not replace DPNH. Furthermore, the addition of a large amount of catalase or catalase with ethanol did not cause any inhibition.

Preliminary experiments⁴ with O_2^{18} and H_2O^{18} indicate that oxygen is directly incorporated from atmospheric oxygen into the carboxyl group of FA and suggest that the primary attack may possibly occur on the C_4 position of ImAA similar to hydroxylation of aromatic compounds. The resulting compound, imidazoloneacetic acid, may be either enzymatically or spontaneously hydrolyzed to FIA as shown in the accompanying diagram.

⁴ Unpublished results by O. Hayaishi, S. Rothberg, and H. Tabor.



The enzyme is therefore an oxygenase and utilizes molecular oxygen for enzymatic oxygenation (35)

SUMMARY

1 A compound was isolated from the aerobic incubation mixture containing β -(imidazolyl-4(5))-acetic acid (ImAA), reduced diphosphopyridine nucleotide, and a partially purified enzyme preparation from ImAA-grown *Pseudomonas*. This compound was identical with synthetic *N*-formimino-aspartic acid (FIA) with respect to the infrared spectra, elementary analysis, the spectrum of color reaction with alkaline ferricyanide-nitroprusside reagent, and R_F values on paper chromatograms.

2 The enzyme, referred to as ImAA oxidase, was purified about 200-fold. Preliminary results indicate that it is an oxygenase and utilizes molecular oxygen for enzymatic oxygenation of ImAA in the presence of reduced diphosphopyridine nucleotide.

3 An enzyme which catalyzes hydrolytic cleavage of FIA to produce formylaspartic acid (FA) and ammonia was partially purified from extracts of *Pseudomonas* sp., which was grown on ImAA as a major carbon and nitrogen source. The product of the enzymatic reaction was isolated in crystalline form and identified as FA by infrared spectra, elementary analysis, and chromatographic behavior on paper and Dowex columns. The properties of the enzyme have been described.

It is a pleasure to acknowledge the technical assistance of James K. Marshall. All the microanalyses were made under the supervision of Dr. W. C. Alford.

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ENZYMATIC CONVERSION OF FORMYLASPARTIC ACID TO ASPARTIC ACID*

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In previous investigations imidazoleacetic acid was shown to be quantitatively converted to formylaspartic acid by a partially purified enzyme preparation obtained from *Pseudomonas* sp. that requires DPNH¹ and oxygen (2, 3). An intermediate in this process was isolated from the incubation mixture with highly purified enzyme preparations and was characterized as *N*-formimino-L-aspartic acid (4). Enzymatic hydrolysis of *N*-formiminoaspartic acid to yield formylaspartic acid and ammonia was catalyzed by a specific enzyme, FIA hydrolase. In all of these previous studies with crude extracts and purified enzyme preparations, FA was accumulated as an end product in a stoichiometric quantity, and formation of free aspartic acid was not detectable even after prolonged incubation.

The purpose of this paper is to present evidence for the further degradation of FA to aspartic acid and formic acid by a specific enzyme, formylaspartic formylase, which is active only in the presence of Fe⁺⁺ or Co⁺⁺.

EXPERIMENTAL

The source of ImAA and FA was previously described (4). Formylglutamic acid was synthesized according to Tabor and Mehler (5). Formyl-DL-leucine was prepared by the method of Fruton and Clarke (6). Acetyl-DL-methionine and acetyl-L-glutamic acid were products of the Nutritional Biochemicals Corporation. Chloroacetyl-L-aspartic acid was prepared by the method of Price *et al.* (7). Formylanthranilic acid and formylkynurenine were kindly furnished by Dr. R. Y. Stanier and Dr. T. Sakan, respectively. *Pseudomonas* sp. (ATCC 11299B) was used throughout this investigation.

* Part of this investigation was supported by grants-in-aid from the National Institutes of Health (No. G-3727). A preliminary report of this work was presented at the 55th general meeting of the Society of American Bacteriologists at New York in May, 1955 (1).

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¹ The following abbreviations are used throughout the paper: ImAA, β -(imidazolyl-4(5))acetic acid, FIA, *N*-formiminoaspartic acid, FA, formylaspartic acid, Tris, tris(hydroxymethyl)aminomethane, DPN, diphosphopyridine nucleotide, DPNH, reduced DPN.

Enzymatic Assay—Determination of amino acids was carried out by a ninhydrin method of Moore and Stein (8), except that citrate buffer at pH 6.2 was used instead of at pH 5.0 in order to prevent spontaneous hydrolysis of labile acylamino acids. The standard assay system (1.0 ml) contained 10 μ moles of substrate, 10 μ moles of Tris buffer, pH 7.0, enzyme, and 1 μ mole of metal ions as indicated in each experiment. After 30 minutes incubation at 37°, an aliquot (0.3 ml) was removed from the reaction mixture and was added to 0.2 ml of 12 per cent cold trichloroacetic acid. The mixture was then centrifuged at $12,500 \times g$ at 0°, and 0.2 ml of the supernatant solution was neutralized with 1.0 N KOH in an ice bath. The total volume was made to 1.0 ml and was used for the ninhydrin method.

1 unit of enzyme is defined as that amount which hydrolyzes 1 μ mole of FA per hour under standard assay conditions, and specific activity is expressed as units per mg of protein. Protein was assayed by the phenol method of Lowry *et al* (9). Aspartic acid was determined by the enzymatic method of Meister *et al* (10), and formic acid was measured by a manometric procedure (11).

Results

Adaptive Nature of Enzymes Splitting FA—In order to study the adaptive response of the organism to various substrates in the growth medium, *Pseudomonas* cells were grown in the following three media: (a) ImAA medium, (b) histidine medium, which, respectively, contained ImAA and histidine as major sources of carbon and nitrogen (4, 12), and (c) a medium containing 0.1 per cent malic acid, 0.1 per cent $(\text{NH}_4)_2\text{SO}_4$, 0.1 per cent Difco yeast extract, 0.15 per cent K_2HPO_4 , 0.05 per cent KH_2PO_4 , and 0.02 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at pH 7.0.

The activity of extracts of the cells (4) was tested with FA as a substrate with the standard assay system. As before, none of these extracts showed appreciable activity when tested without addition of metal ions. This is also consistent with the observation by Tabor and Mehler that extracts from histidine-adapted *Pseudomonas* did not degrade FA (5). Upon addition of various metal ions to the incubation mixture, the presence of 0.001 M CoCl_2 was found to stimulate the activity of all three extracts. A more than 10-fold increase was observed with extracts of histidine-grown cells or ImAA-grown cells. Extracts of malate-grown cells also showed considerable activity in the presence of Co^{++} (Table I). In contrast to cobaltous ion, however, addition of ferrous ion stimulated the activity of extracts from ImAA-grown cells only, suggesting the presence of a specific adaptive enzyme in the ImAA-grown cells.

Since these results indicated the presence of different formylases in extracts of ImAA-grown cells and histidine-grown cells, purification of the

two activities was carried out with different assay procedures, the assay of FA formylase from histidine-adapted cells was performed in the presence of Co^{++} , and that of ImAA-grown cells was carried out in the presence of Fe^{++}

Purification of Formylaspartic Formylase from Extracts of ImAA-Grown Cells (Formylase I)—For each 33 ml of crude extract 9.1 ml of a 1 per cent

TABLE I
Adaptive Nature of Organism

The incubation mixture (1.0 ml) contained 0.1 ml of crude extracts (0.5 mg of protein), 10 μmoles of formylaspartic acid, 20 μmoles of Tris buffer, pH 7.0, 2 μmoles of cobaltous ions as CoCl_2 , or ferrous ions as FeSO_4 as indicated. Incubation was for 1 hour at 37° . The numbers represent specific activities.

Addition	Medium		
	Malate	Histidine	ImAA
None	0.5	0.4	0.7
Co^{++}	2.6	6.3	6.0
Fe^{++}	0.5	0.4	5.2

TABLE II
Purification of Formylase I from ImAA-Adapted Cells

Standard assay system with 1 μmole of FeSO_4

Enzyme	Specific activity	Total activity	Total yield
	<i>units per mg protein</i>	<i>units</i>	<i>per cent</i>
Crude extract	5.1	188	100
Protamine fraction	7	143	76
Fraction I (30–40% $(\text{NH}_4)_2\text{SO}_4$)	8	16	8.5
“ II (40–50% $(\text{NH}_4)_2\text{SO}_4$)	44	58	31
“ III (50–70% $(\text{NH}_4)_2\text{SO}_4$)	15	12	6.5
Aluminum hydroxide gel fraction	99	24	18

solution of protamine sulfate (Eli Lilly and Company) were added. After 15 minutes at 0° , the precipitate was centrifuged, and for each 40 ml of the supernatant solution 8.4 gm of $(\text{NH}_4)_2\text{SO}_4$ were added. After 30 minutes at 0° , the precipitate was centrifuged and 2.8 gm of $(\text{NH}_4)_2\text{SO}_4$ were added to the supernatant fluid. The precipitate was collected by centrifugation and dissolved in 0.02 M phosphate buffer, pH 7.1 (Fraction I, Table II). To the supernatant solution 2.8 gm of $(\text{NH}_4)_2\text{SO}_4$ were added. The precipitate was collected by centrifugation and dissolved in the same buffer (Fraction II). 5.6 gm of $(\text{NH}_4)_2\text{SO}_4$ were added to the supernatant fluid

The precipitate was collected by centrifugation and dissolved in 0.02 M phosphate buffer, pH 7.0 (Fraction III). Each fraction was dialyzed against distilled water at 5° for 10 hours. 2.0 ml of aluminum hydroxide gel (18.5 mg of solids per ml) were centrifuged, and 10 ml of dialyzed fraction II were added to the semisolid gel. The mixture was stirred for about 20 minutes at 0°. After centrifugation, the gel was eluted with 5.0 ml of 0.04 M phosphate buffer, pH 7.1, for about 15 minutes at 0°, and centrifuged. The enzyme was dialyzed against cold distilled water for 4 hours.

Purification of Formylaspartic Formylase from Histidine-Grown Cells (Formylase II)—To 160 ml of crude extract were added 40 ml of a 1 per

TABLE III

Purification of Formylase II from Histidine-Adapted Culture

Standard assay system with 1 μ mole of CoCl_2

Enzyme	Specific activity	Total activity	Total yield
	units per mg protein	units	per cent
Crude extract	5.1	250	100
Protamine fraction	10.6	240	96
Fraction I (35–45% $(\text{NH}_4)_2\text{SO}_4$)	7.5	11	4.4
“ II (45–55% $(\text{NH}_4)_2\text{SO}_4$)	6	13	5.2
“ III (55–65% $(\text{NH}_4)_2\text{SO}_4$)	31.6	62	26.0
“ IV (65–80% $(\text{NH}_4)_2\text{SO}_4$)	44.0	87	36.0
Acetone fraction	62	117	47.7
Aluminum hydroxide gel fraction	350	100	40.4

cent protamine solution (Eli Lilly and Company). After 15 minutes at 0°, the precipitate was removed by centrifugation. To 200 ml of the supernatant solution were added 48.8 gm of $(\text{NH}_4)_2\text{SO}_4$. After 30 minutes at 0°, the precipitate was centrifuged (Fraction I, Table III), and 14.0 gm of $(\text{NH}_4)_2\text{SO}_4$ were added to the supernatant fluid. The precipitate was collected by centrifugation (Fraction II). To the supernatant solution, 14 gm of $(\text{NH}_4)_2\text{SO}_4$ were added. After 30 minutes, the precipitate was collected (Fraction III). To the supernatant fluid, 14.0 gm of $(\text{NH}_4)_2\text{SO}_4$ were added. After 15 minutes, the precipitate was collected (Fraction IV).

Each fraction collected by centrifugation was dissolved in 20 ml of 0.02 M phosphate buffer, pH 7.0. In contrast to formylase I, Fractions III and IV (Table III) which were precipitated between 55 and 80 per cent saturation of ammonium sulfate contained most of the activity, whereas only a small amount of activity was detected in Fractions I and II.

Fractions III and IV were combined and dialyzed against cold distilled water for 6 hours. To 18 ml of the dialyzed enzyme solution were added 2 ml of 1 M sodium acetate, and then 10.7 ml of acetone were added dropwise with mechanical stirring at -10° . After standing for 5 minutes, the precipitate was removed by centrifugation, and 5.3 ml of acetone were added to the supernatant fluid in a similar manner. After 5 minutes at -10° , the precipitate was collected by centrifugation and dissolved in 5 ml of cold water (acetone fraction).

2.5 ml of aluminum hydroxide gel were centrifuged, 10 ml of acetone fraction were added to the semisolid gel, and the stirred mixture was left

TABLE IV
Substrate Specificity of Formylases I and II

Formylase I (0.08 mg of protein) or formylase II (0.02 mg of protein), 10 μ moles of substrate, 10 μ moles of Tris buffer, pH 7.2, 2 μ moles of Co^{++} , or 1 μ mole of Fe^{++} as indicated, in a total volume of 1.0 ml. Incubation was for 30 minutes at 37° . The numbers represent micromoles of substrate decomposed per hour per mg of protein.

Enzyme	Metal	FA	Chloroacetyl L-aspartic acid	Formylglutamic acid	Acetyl-L-glutamic acid
Formylase I	None	32	0	5	0
	Fe^{++}	100	0	9	0
	Co^{++}	51	92	8	7
Formylase II	None	26	0	340	80
	Fe^{++}	6	0	350	
	Co^{++}	330	250	350	110

at 0° for about 20 minutes. After centrifugation, the supernatant solution was discarded. The gel was washed once with 5.0 ml of 0.04 M phosphate buffer, pH 7.1, and then eluted with 5.0 ml of 0.2 M phosphate buffer, pH 7.0. The enzyme preparation was dialyzed against cold distilled water for 4 to 6 hours before testing, since phosphate buffer binds metal ions, particularly ferrous ions.

Metal Requirement and Substrate Specificity of Formylases I and II—A partially purified preparation of formylase I appeared to be specific for FA. The activity towards FA was stimulated by the addition of ferrous ions and also by cobaltous ions to a lesser extent. Formyl- and acetylglutamic acids were only slightly metabolized by this preparation (Table IV).

On the other hand, the purified formylase II preparation showed much higher activity towards formyl- and acetylglutamic acids, and the activity

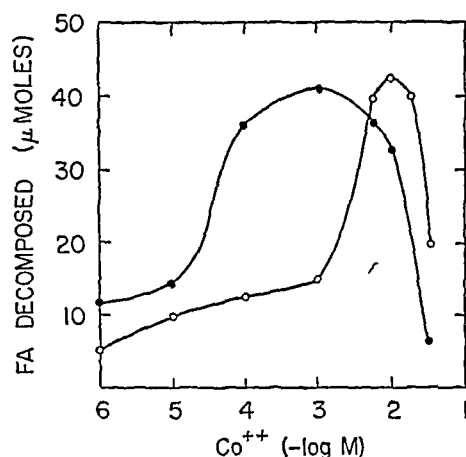


FIG 1

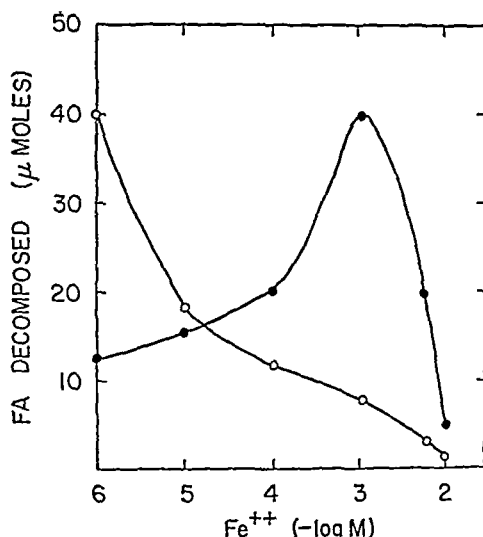


FIG 2

FIG 1 Activity of formylases I and II with varying concentration of cobaltous ions. Formylase I (0.08 mg of protein) or formylase II (0.02 mg of protein), 10 μ moles of FA, 10 μ moles of Tris buffer, pH 7.4, the amount of CoCl_2 as indicated, in a total volume of 1.0 ml. Incubation was for 30 minutes at 37° . ●, formylase I, ○, formylase II.

FIG 2 Activity of formylases I and II with varying concentration of Fe^{++} ions. Formylase I (0.08 mg of protein) or formylase II (0.02 mg of protein), 10 μ moles of FA, 10 μ moles of Tris buffer, pH 7.4, varying concentrations of ferrous ions ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), in a total volume of 1.0 ml. Incubation was for 30 minutes at 37° . ●, formylase I, ○, formylase II.

TABLE V
Products of Reaction

The incubation mixture (2.0 ml) contained formylase I (0.89 mg of protein), 100 μ moles of FA, 50 μ moles of Tris buffer, pH 7.5, 1 μ mole of Fe^{++} , and 1 μ mole of sodium ascorbate*. Incubation was for 5 hours at 37° . The incubation mixture for formylase II contained, in 2.0 ml, 100 μ moles of FA, 5 μ moles of Co^{++} , enzyme (0.6 mg of protein), 50 μ moles of Tris buffer, pH 7.5. Incubation was for 6 hours at 37° .

Formylase	$-\Delta \text{FA}^\dagger$	$\Delta \text{aspartic acid}^\dagger$	$\Delta \text{formic acid}$
	μmoles	μmoles	μmoles
I	90.0	88.0	86.5
II	96.2	96.0	92.0

* Sodium ascorbate was added in order to keep ferrous ions in reduced form.

† Ninhydrin assay

‡ Enzymatic assay

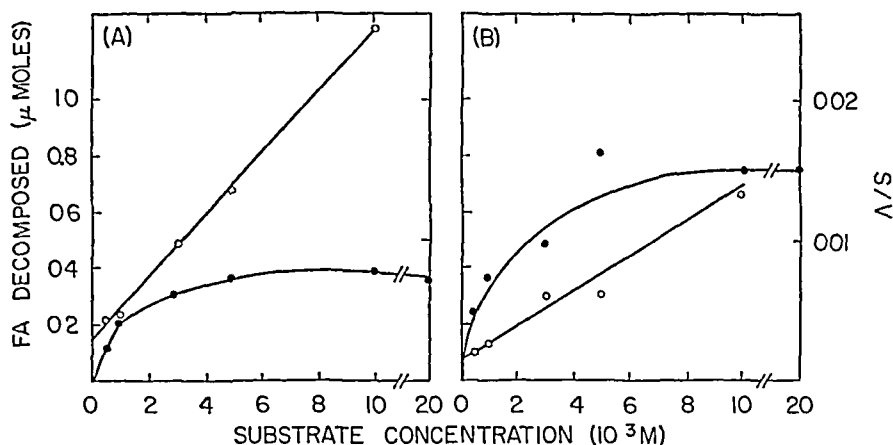


FIG 3 Effects on the reaction rate of varying concentrations of FA A, incubation mixture (1.0 ml) contained formylase I (0.06 mg), 1 μmole of FeSO_4 , 10 μmoles of Tris buffer, pH 7.5, and FA as indicated. Incubation was for 30 minutes at 37°. B, incubation mixture (1.0 ml) contained 0.04 mg of formylase II, 1 μmole of CoCl_2 , 10 μmoles of Tris buffer, pH 7.5, and FA as indicated. Incubation was for 30 minutes at 37°.

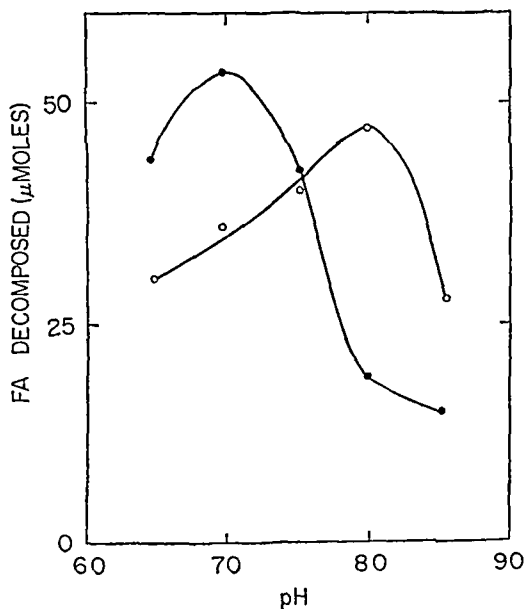


FIG 4 Rate of the reaction as a function of pH. Formylase I (0.08 mg of protein) or formylase II (0.02 mg of protein), 10 μmoles of FA, 10 μmoles of Tris buffer, pH 7.5 to 8.6, or 10 μmoles of acetate buffer, pH 5.0 to 6.5, 1 μmole of Fe^{++} with formylase I or 2 μmoles of Co^{++} with formylase II. Incubation was for 30 minutes at 37°. ●, formylase I, ○, formylase II.

towards FA was stimulated by the addition of Co^{++} but was suppressed in the presence of Fe^{++}

Both enzymes had some activity towards chloroacetylaspartic acid in the presence of cobaltous ions, but acetylmethionine, formylleucine, formylkynurenine, and formylanthranilic acid were all inert

The activity of the formylases as a function of cobaltous ion concentration is shown in Fig 1

The optimal concentration of Co^{++} for formylase I was approximately 10^{-3} M, while that for formylase II is around 10^{-2} M

Fig 2 shows the response of the two enzymes towards ferrous ions Formylase II is increasingly inhibited by ferrous ions as the concentration increases above 10^{-5} M In contrast, formylase I shows marked stimulation and the optimal concentration lies around 10^{-3} M

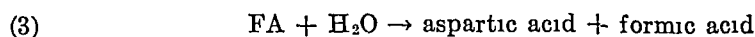
Mn^{++} ions (10^{-3} M) stimulated formylase II approximately 150 per cent but failed to affect formylase I activity at 10^{-3} or 10^{-4} M Cd^{++} , Zn^{++} , and Ni^{++} were all ineffective

Products of Reaction—Table V shows that both formylase I and formylase II catalyze the simple hydrolysis of FA and that equivalent amounts of L-aspartic acid and formic acid were produced from formyl-L-aspartic acid

Effects of Substrate Concentration and pH on Reaction Rate—The effects on the reaction rate of varying concentrations of FA are shown in Fig 3 K_m values are calculated from these data to be 1.32×10^{-3} M and 1.25×10^{-3} M for formylases I and II, respectively The optimal pH of formylase I is approximately 7.0, while that of formylase II is around pH 8.0 (Fig 4)

DISCUSSION

It may be concluded from these and previous studies (2-4) that the metabolic pathway of ImAA in *Pseudomonas* involves the accompanying reactions



A specific formylase, which was purified from ImAA-grown cells of *Pseudomonas*, catalyzes the hydrolysis of FA in the presence of Fe^{++} or Co^{++} (Reaction 3) It also acts upon chloroacetylaspartic acid in the presence of Co^{++} , but formyl and acetyl derivatives of other amino acids were not metabolized This enzyme is referred to as formylaspartic formylase

Another enzyme, tentatively designated formylase II, was purified from histidine-grown cells of *Pseudomonas* Although this enzyme is able to catalyze hydrolysis of FA in the presence of cobaltous ions, it exhibits much higher activity towards acyl derivatives of glutamic acid and is prob-

ably identical with the enzyme described by Taboi and Mehler (5) More recently Kato *et al* (13) described a similar enzyme from *Pseudomonas* and named it formylglutamic deformylase

The evidence presented in this paper shows that these two enzymes, both of which hydrolyze FA under certain conditions, exhibit different substrate specificities, metal requirements, and physicochemical properties

Both enzymes differ from hitherto known acylases, such as acylases I and II, which were isolated from animal cells by Rao and his collaborators (14) and were shown to exhibit much less substrate specificity They differ also from formylkynurenine formylase previously reported by Mehler and Knox (15) and by Hayaishi and Stanier (16)

Recent studies on adaptive enzyme formation have shown that a single activity can be elicited by a large variety of inducers of related chemical structure For example, β -D-galactosidase of *Escherichia coli* could be induced by various compounds having the β -galactoside linkage (17) In such studies the induced activity is usually determined with intact cells, with toluene-treated cells, or with crude extracts With these techniques alone it is not always clear whether all the inducers evoke the formation of the same enzyme or whether different enzymes with a common activity are formed in response to different inducers

In the present work the use of more refined enzymological techniques has uncovered an instance of the latter type Formylases I and II are formed by the same type of cell in response to different inducers and have in common the ability to degrade FA into formic and aspartic acids Yet they are distinct enzymes which exhibit different substrate specificities, metal cofactor requirements, and physicochemical properties

SUMMARY

1 The metabolism of formylaspartic acid was studied with partially purified enzyme preparations obtained from β -(imidazolyl-4(5))acetic acid (ImAA)-grown cells and histidine-grown cells of *Pseudomonas*

2 The two kinds of cells yielded two different enzymes, both of which catalyze the hydrolysis of formylaspartic acid to yield formic acid and aspartic acid

3 Formylaspartic acid formylase, which was obtained from ImAA-grown cells, is more specific and is stimulated by Fe^{++} and, to a lesser extent, by Co^{++} Another formylase prepared from histidine-grown cells could hydrolyze acyl derivatives of aspartic as well as of glutamic acid, and its activity towards formylaspartic acid was stimulated only in the presence of Co^{++} ions

4 Possible implications of these findings with regard to adaptive enzyme studies were discussed

We wish to thank Dr Robert De Mars for his kind advice and aid in the preparation of the manuscript

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STUDIES ON THE PROPERTIES OF THREONINE ALDOLASES*

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Braunshtein and Vilenkina (2), in studies on the formation of glycine from serine, threonine, and other hydroxyamino acids in animal tissues, reported the presence of an enzyme which formed acetaldehyde and glycine from threonine and allothreonine. In subsequent work by Lin and Greenberg (3) it was shown that allothreonine is decomposed about twenty times faster than threonine in crude homogenates, that equimolar amounts of acetaldehyde and glycine are formed from threonine and allothreonine, and that the D isomers are not decomposed. More recently Gilbert (4) found threonine to be synthesized from acetaldehyde and glycine upon incubation with a soluble cell-free rat liver enzyme.

In the present work evidence has been obtained that allothreonine and threonine are attacked by separate enzymes. A method for the preparation of the enzymes has been developed and a study has been made of some of their properties.

Because of the close analogy of the enzymatic reactions to aldol condensations, the enzyme which catalyzes the decomposition of threonine will continue to be referred to as threonine aldolase, and that which catalyzes the decomposition of allothreonine as allothreonine aldolase.

Materials and Methods

DL-Threonine, 0.5 M (Schwarz Laboratories, Inc., allo-free), adjusted to pH 7.6 with NaOH and stored in the cold.

DL-Allothreonine, 0.5 M (Nutritional Biochemicals Corporation), prepared as above.

Pyridoxal phosphate, 10^{-3} M (California Foundation for Biochemical Research), dissolved in distilled water and stored frozen.

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Acetaldehyde, 0.2 M (Eastman Kodak Company), prepared by dissolving 1.13 ml of redistilled acetaldehyde in 100 ml of distilled water

Periodate reagent, 0.5 M (G. Frederick Smith Chemical Company), dissolved in distilled water and prepared fresh each week

Semicarbazide reagent, 0.1876 gm of semicarbazide hydrochloride (Eastman Kodak Company), dissolved in 250 ml of 0.16 M phosphate buffer, pH 7.0

Enzyme Assay Methods

Method I Acetaldehyde Determination—The reaction was carried out in the modified, all-glass Conway diffusion cells described by Lin and Greenberg (3), and the acetaldehyde was determined by the method of Burbidge *et al* (5)

3.0 ml of semicarbazide reagent were carefully pipetted into the central well of the diffusion cell. The test solutions pipetted into the outer well were 0.1 ml of 10^{-3} M pyridoxal phosphate,¹ 0.25 ml of DL-threonine or DL-allothreonine solutions, and 1.65 ml of 10^{-3} M phosphate buffer, pH 7.6. At zero time, 0.5 ml of properly diluted enzyme solution was added to the outer well, the ground glass stopper was inserted, and the cells were incubated in a Dubnoff shaking incubator for 30 minutes. To stop the reaction, the flasks were placed in a water bath at 80–90° for 2 minutes. They were then returned to the shaking apparatus for 90 minutes to complete the diffusion of the acetaldehyde.

To determine the quantity of acetaldehyde produced, 2.0 ml of the semicarbazide solution were removed from the central well and diluted to 10.0 ml with distilled water. The optical density of this solution was measured at 224 m μ in a Beckman DU spectrophotometer in a 1.0 cm cell. The blank solution consisted of 2.0 ml of the semicarbazide reagent diluted to 10.0 ml with distilled water, the control flasks contained all the reaction components except the substrate. The production of acetaldehyde was proportional to the amount of enzyme at values below 10 μ moles (see Fig. 1).

Method II Determination of Formed Threonine—In this assay procedure the reaction was carried out in 2.1 ml dram vials fitted with a 2 mm polyethylene seal. A standard screw cap was placed over the polyethylene seal to hold it in place and to produce an air-tight closure. All the solutions were added to or removed from the vials with a hypodermic syringe. The reaction medium consisted of 0.2 ml of acetaldehyde, 0.25 ml of glycine, and phosphate buffer to give a total volume of 2.1 ml after the addi-

¹ The abbreviations used are PLP, pyridoxal phosphate, PCMB, *p*-chloromercuribenzoate, BAL, dithiopropanol, EDTA, ethylenediaminetetraacetic acid, ATP, adenosine triphosphate

tion of the enzyme At zero time, 0.5 ml of properly diluted enzyme solution was added, together with a glass bead, and the vials were sealed with the polyethylene disk The vials were then placed horizontally in a Dubnoff metabolic shaking incubator and incubated for 30 minutes To stop the reaction, the contents of the vials were rapidly transferred to centrifuge tubes which contained 0.5 ml of 10 per cent trichloroacetic acid The tubes were then heated in a metal heating block at 90° for 30 minutes to remove substrate acetaldehyde which would interfere with subsequent threonine and allothreonine determinations The protein was removed

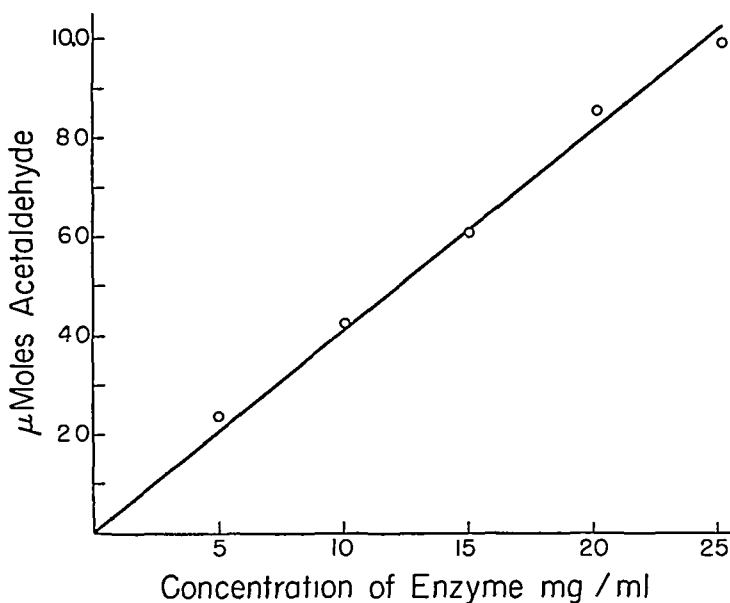


Fig 1 Formation of acetaldehyde as a function of the enzyme concentration

by centrifugation, and the supernatant fluid was diluted to 10.0 ml with distilled water

The threonine and allothreonine present were determined by cleavage with periodate and the acetaldehyde liberated The acetaldehyde could be determined as in Method I by diffusion However, a more rapid procedure was developed by determining the acetaldehyde colorimetrically with 2,4-dinitrophenylhydrazine

To carry out the determination, a suitable aliquot of the incubation mixture was placed in a 10 ml volumetric flask, and the pH was adjusted to the end point of methyl red with 6 M NaOH, 0.15 ml of 0.5 M periodic acid was added, the flasks were stoppered, and the reaction was allowed to go to completion (5 minutes) To remove the excess of periodic acid, a saturated solution of SnCl_2 in 2 N HCl was added until a clear, colorless

solution was obtained 0.1 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl was next added to form the hydrazone. After 5 minutes standing, 2.0 ml of 95 per cent ethanol were added to prevent crystallization of the hydrazone, and the color was developed by diluting the mixture to 10 ml with 2.5 N NaOH. The optical density of the resulting solution was measured in exactly 15 minutes with a Beckman model B spectrophotometer at 515 m μ . The intensity of the color produced was proportional to the threonine or allothreonine concentration between 0.10 and 1.0 μ mole (Fig. 2).

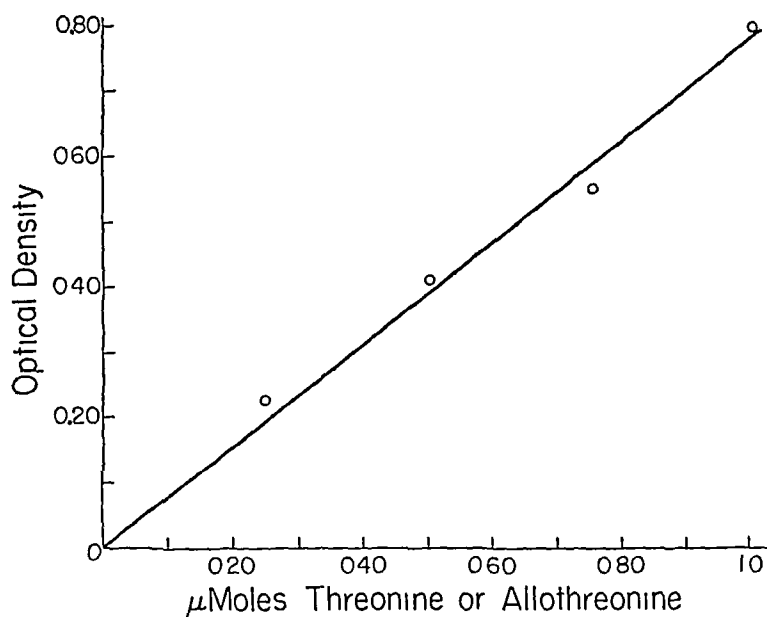


FIG. 2 Proportionality between threonine and allothreonine concentrations and optical density in Method II by the determination of formed threonine

Enzyme Solutions—The enzyme solutions for assay were diluted with cold phosphate buffer to contain 10 to 50 mg of protein, depending on the activity.

Enzyme Unit—An enzyme unit was defined as the quantity of enzyme which produced 1.0 μ mole of acetaldehyde or glycine in 0.5 hour, at 37°. The specific activity was defined as the enzyme units per optical density unit of protein.

Protein Determination—Protein was determined by the ultraviolet absorption at 278 m μ in a 1.0 cm cuvette (6). The extinction coefficient readings were converted to mg of dry protein by multiplying by the factor 1.10. This value was obtained by analysis of dialyzed, lyophilized enzyme preparations.

Results

Distribution of Enzyme Activity—A comparison of the enzyme activity in crude homogenates of the liver and kidney of a number of mammalian

TABLE I

Comparison of Specific Activities and Activation with PLP of Threonine and Allothreonine Aldolase of Various Tissues

Reaction components 1.0 ml of tissue homogenates, 125 μ moles of DL-threonine or DL-allothreonine, 0.1 M phosphate buffer, pH 7.6, to give a volume of 2.5 ml per flask

Enzyme source	Substrate	PLP	Enzyme activity, specific activity $\times 100$
Beef liver	Threonine	—	0.02
	"	+	0.02
	Allothreonine	—	2.6
Rabbit liver	"	+	4.5
	Threonine	—	0.25
	"	+	0.25
" kidney	Allothreonine	—	3.8
	"	+	5.2
	Threonine	—	0.0
" kidney	"	+	0.0
	Allothreonine	—	0.45
	"	+	1.3
Rat liver	Threonine	—	0.1
	"	+	0.1
	Allothreonine	—	1.5
" kidney	"	+	1.5
	Threonine	—	0.0
	"	+	0.0
" kidney	Allothreonine	—	0.2
	"	+	0.2
Horse liver	Threonine	—	0.25
	"	+	0.25
	Allothreonine	—	2.10
" kidney	"	+	3.80
	Threonine	—	0.3
	"	+	0.3
Sheep "	Allothreonine	—	6.1
	"	+	6.2

species and the relative activation with PLP is presented in Table I. All the livers exhibited enzyme activity, with sheep liver showing the highest specific activity. This was used subsequently as the enzyme source in the purification studies. Both enzymes were also present in yeast, but

the specific activity of yeast cells disrupted by sonic treatment was considerably lower than that of mammalian tissues

Preparation of Threonine Aldolases

Liver—Fresh liver was obtained from the abattoir, cooled with ice, washed in cold water, and freed from fat and connective tissue. The livers were cut into 15 gm sections, wrapped in Parafilm or tin foil, and frozen and stored at -20° until used.

Extraction—250 gm of frozen liver were mixed with 250 ml of 0.1 M phosphate buffer, pH 7.6, which contained 0.1 M NaF,² and warmed to 0° . The mixture was then homogenized in a Waring blender for 2 minutes.

Controlled Heat Denaturation of Non-Threonine Aldolase Protein—The crude homogenate was adjusted to pH 7.6 with 1 N NaOH and heated to 55° in a hot water bath. This temperature was maintained for 5 minutes. After cooling in an ice bath to 0° , the inactive precipitate was removed by centrifugation at 0° .

Ammonium Sulfate Precipitation—The clear red, cooled supernatant liquid from the heated extract was brought to 30 per cent saturation by the slow addition of solid ammonium sulfate.³ The pH of the extract was maintained at 7.6 during the addition of the ammonium sulfate, and the precipitate was removed by centrifugation at 0° .

Ammonium Sulfate Extraction—A graded series of ammonium sulfate solutions of 20 to 50 per cent saturation was prepared by the appropriate dilution with distilled water of a saturated ammonium sulfate solution,⁴ pH 7.6. The protein fraction which was not soluble in 30 per cent saturated ammonium sulfate was then successively extracted with the graded ammonium sulfate solutions of 50, 45, and 35 per cent saturation in the following manner. The protein precipitate was extracted with 5 times its weight of the highest ammonium sulfate concentration (50 per cent), and the proteins not soluble in 50 per cent ammonium sulfate were separated by centrifugation. The extracted soluble proteins were reprecipitated without delay by the addition of saturated ammonium sulfate, pH 7.6, and were centrifuged, tested for activity, and stored at -20° . This procedure was repeated with ammonium sulfate solution of 45 and 35 per cent

² Fluoride was found to protect the enzymes in crude homogenates, possibly by inhibiting the decomposition of PLP by phosphatases. After the first fractionation with ammonium sulfate, fluoride was ineffective in protecting against inactivation. In crude preparations, addition of PLP also reduced the progressive inactivation of the enzymes.

³ The formula of Kunitz (7) was employed to calculate per cent saturation of ammonium sulfate.

⁴ Prepared by adding ammonium sulfate to saturation with stirring, adjusting to pH 7.6 with concentrated ammonia, and filtering off the excess salt.

saturation The enzyme fraction soluble in 40 per cent saturated ammonium sulfate usually contained the aldolases A summary of the purification data is given in Table II

Attempts at Further Purification—The threonine aldolases were found to be highly labile and very resistant to further attempts to separate and purify them Exploitation with other salts demonstrated that sodium citrate was superior for purification to ammonium sulfate However, the greater cost and limited improvement in the purification did not justify the use of citrate

No success in purification was obtained with the organic solvents, acetone, butanol, ethanol, and methyl Cellosolve This was also true of the

TABLE II
Summary of Yields and Specific Activities Obtained during Purification of Threonine Aldolases

Fraction	Volume	Total protein	Enzyme units		Specific activity $\times 100$		Activity ratio, threonine/allothreonine
			Threonine	Allothreonine	Threonine	Allothreonine	
Crude homogenate	330	58,000	296	6200	0.51	10.7	0.048
Supernatant, after heating to 55°	175	17,500	192	3100	1.10	17.7	0.062
Ammonium sulfate ppt, 30%	25	3,260	75	670	2.3	20.5	0.11
Ammonium sulfate extract, 40%	10	123	21	240	17.1	195	0.088

adsorbents, alumina, calcium phosphate gel, bentonite, and barium sulfate Gradient elution chromatography by the method of Schwimmer (8) was unsuccessful because of the extreme lability of these enzymes, particularly in high dilutions

Evidence for Presence of Two Enzymes

Changes in Ratio of Threonine to Allothreonine Decomposition with Purification—The ratios of the specific activities of the aldolases were followed during the preparation of the enzymes From Table II it can be seen that the ratios of the quantities of threonine and allothreonine decomposed were not constant

Changes in Ratio of Threonine to Allothreonine Decomposition in Presence of Structural Analogues—Compounds with functional groups sterically related to threonine and allothreonine inhibited the enzymes differentially

Serine, homoserine, Chloromycetin, and *threo*- and *erythro*-serinol were tested. The extent of inhibition by each is shown in Table III.

Other Evidence—This is provided by a variety of observations on the different responses in the decomposition of threonine and allothreonine by enzyme extracts. A wide variation was found in the magnitude of the activation with PLP for the two substrates. An extreme case was one sheep liver preparation in which PLP produced no increase in the rate of decomposition of threonine, although the rate for allothreonine was increased 10-fold.

TABLE III

Effect of Structural Analogues on Threonine and Allothreonine Aldolase Activity

Reaction components: 80 mg of enzyme protein, 0.10 μ mole of PLP, 125 μ moles of DL-allothreonine, 100 μ moles of analogue, phosphate buffer to give a volume of 3.0 ml per flask.

Substrate	Analogue	Enzyme activity	Activity ratio, threonine allothreonine
		<i>enzyme units</i>	
Threonine		1.0	0.09
Allothreonine		11.5	
Threonine	<i>erythro</i> -Serinol	0.5	0.02
Allothreonine	"	2.2	
Threonine	<i>threo</i> -Serinol	1.0	0.40
Allothreonine	"	2.5	
Threonine	Serine	0.4	0.64
Allothreonine	"	6.3	
Threonine	Homoserine	0.6	0.85
Allothreonine	"	7.2	
Threonine	Chloromycetin	1.1	0.10
Allothreonine	"	10.2	

With increasing time of incubation up to 2 hours, the rate of decomposition of allothreonine remained nearly constant, while that of threonine was progressively slowed down. The rate of decomposition of allothreonine increased nearly linearly with increasing enzyme concentration over about a 20-fold range, but that rate for threonine did not increase any further after the enzyme concentration was increased 4-fold with the same liver preparation.

Properties of Threonine Aldolases

Activation with PLP—Activation by PLP in crude extracts is demonstrated in Table I. In the purified preparations, the increase in enzyme activity as a function of the PLP concentration followed Michaelis-Menten

kinetics The data plotted according to the method of Lineweaver and Burk (9) yielded straight lines From these there were calculated the K_s values for PLP of 1.11×10^{-4} M for threonine aldolase and 1.74×10^{-6} M for allothreonine aldolase No other vitamin B₆ derivative would replace PLP

Effect of —SH Reagents—PCMB completely inhibited the enzyme activ-

TABLE IV

Inhibition and Reactivation of Threonine Aldolases

Reaction components 30 mg of enzyme protein, 125 μ moles of DL-threonine or DL-allothreonine, 0.1 μ mole of PLP, 10^{-3} M phosphate buffer, pH 7.6, to give a volume of 2.5 ml per flask

Substrate	Reagents	Enzyme activity
		<i>enzyme units</i>
Threonine	None	0.6
Allotreonine	"	2.4
Threonine	PCMB, 10 μ moles	0.0
Allotreonine	" 10 "	0.0
Threonine	Iodoacetate, 10 μ moles	0.4
Allotreonine	" 10 "	2.2
Threonine	None	0.7
Allotreonine	"	3.1
Threonine*	O-Iodosobenzoate, 50 mg	0.1
Allotreonine	" 50 "	2.1
Threonine*	BAL, 50 mg	0.7
Allotreonine	" 50 "	3.1
Threonine*	O-Iodosobenzoate† + BAL†	0.65
Allotreonine	" "	3.2

* In these experiments, 1 gm of enzyme protein was oxidized with 10 μ moles of O-iodosobenzoate for 0.5 hour at pH 7.6 and 4°. An aliquot was removed and the remaining enzyme reduced with 100 μ moles of BAL for 0.5 hour at pH 7.6 and 4°

† 50 mg

ity, as did mercury and copper ions, while iodoacetate exhibited only a minimal inhibitory effect (Table IV) The enzyme also was inhibited by oxidation with iodosobenzoate, but this could be prevented or completely reversed by BAL (Table IV) It was also observed repeatedly that enzyme preparations which lost enzyme activity upon prolonged aging could be partially reactivated by glutathione or cysteine These findings support the view that the threonine aldolases are —SH enzymes

Optimal pH—The effect of pH on the sheep liver enzyme activity was studied with both amino acid substrates in the presence of PLP, and for both the forward splitting reaction and the synthetic reaction with acetal-

dehyde and glycine as substrates (Fig 3) The optimal pH was the same for both enzymes, whether the forward or the reverse reaction rate was measured, namely $\text{pH } 7.6 \pm 0.2$ This same value was previously found for the rat liver threonine aldolase (3) and for guinea pig liver threonine aldolase (2)

Stoichiometry—Incubation of L-threonine and L-allothreonine with the enzyme preparation resulted in equivalent decreases of the two substrates and the formation of corresponding amounts of acetaldehyde and glycine (Table V) The decrease in threonine or allothreonine was measured by

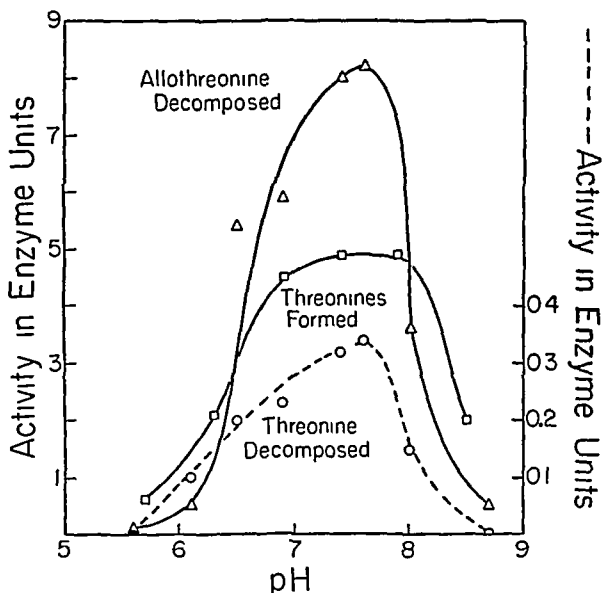


FIG 3 pH-activity curves of threonine aldolases The curves show that the optimal pH is the same for both the decomposition of the threonines and for their re-synthesis

the periodate reaction, the acetaldehyde formed by the usual assay method, and the glycine formed by cleavage with ninhydrin and estimation of the formaldehyde produced with chromotropic acid (10)

Substrate Affinity—The enzyme-substrate dissociation constants for threonine and allothreonine were determined in the usual manner from Lineweaver-Burk plots The DL forms of the amino acids were employed in the tests because of the unavailability of the resolved amino acids, but the constants were calculated for the L-amino acids, since the D forms are not enzymatically cleaved (3) The K_s values found for threonine and allothreonine as substrates were $69 \times 10^{-3} \text{ M}$ and $4.35 \times 10^{-3} \text{ M}$, respectively The K_s values for the reaction of PLP with these enzymes are given above

In studies with a rat liver preparation, Lin and Greenberg obtained K_s figures of 25×10^{-3} for L-threonine and 1×10^{-3} for L-allothreonine

Failure to Show Other Cofactor Requirements—No inhibitory effect on the aldolases was observed after treatment of the enzymes with EDTA, 2,2-bipyridine, 8-hydroxyquinoline, ascorbic acid, or BAL. Likewise, no activation of the aldolases was obtained after the addition of all metals⁵ of physiological importance, or of chromium or aluminum.

PLP and tetrahydrofolic acid have been found to be cofactors for the reaction of the formation of serine from glycine and formaldehyde (11). It was not possible in our work to show the presence of cofactors other than PLP for the threonine aldolases. Materials which were tested for

TABLE V
Formation of Equivalent Amounts of Acetaldehyde and Glycine from Threonine and Allothreonine

Reaction components 10 μ moles of DL-threonine or DL-allothreonine, 30 mg of enzyme protein, 0.1 μ mole of PLP, 10^{-3} M phosphate buffer, pH 7.6, to give a volume of 2.5 ml per flask

Initial			Final		
Substrate	Acetaldehyde	Glycine	Substrate	Acetaldehyde	Glycine
	μ mole	μ mole		μ moles	μ moles
Threonine, 10 μ moles	0.0	0.0	Threonine, 9.7 μ moles	0.18	0.21
Allothreonine, 10 μ moles	0.0	0.0	Allothreonine, 7.9 μ moles	2.0	2.1

both the synthesis and the breakdown of threonine and allothreonine were extracts of yeast and liver, folic acid and ATP, citrovorum factor and ATP, tetrahydrofolic acid and ATP, coenzyme A and reduced diphosphopyridine nucleotide, and acetyl phosphate.

Test for Other Substrates—A number of naturally occurring hydroxy compounds and aldehydes were tested to determine whether they could serve as substrates for the partially purified threonine aldolases. This possibility was indicated by the fact that Braunshtein and Vilenkina (2) observed the formation of glycine from a number of hydroxyamino acids, and that Gilbert (12) has reported the decomposition of *threo*- and *erythro*-phenylserine to benzaldehyde and glycine by crude liver extracts. Formaldehyde, propionaldehyde, and acetone possessed no activity when incu-

⁵ The activity of the threonine aldolases was found to be markedly increased (about doubled) at lowered ionic strengths of the medium. The reduction in activity with increased ionic strength could be counteracted by preincubation with PLP.

bated with glycine, PLP, and the purified enzymes Serine, homoserine, epinephrine, alanine, lactic acid, glycolic acid, and phosphothreonine produce no detectable volatile carbonyl decomposition products

The effect of substrate concentration on the activity of the enzymes was investigated with acetaldehyde. A rather narrow range of concentration was found for optimal enzyme activity, and the reaction rate fell off quite rapidly when the optimal concentration (0.017 M) was exceeded. Acetaldehyde concentrations in excess of 0.02 M produced visible denaturation of the protein solution.

TABLE VI

Determination of Apparent Equilibrium Constant of Allothreonine Formation

Experiment No	Allothreonine, $M \times 10^3$		Glycine, $M \times 10^3$		Acetaldehyde, $M \times 10^3$		K_{eq}
	Initial	Final	Initial	Final	Initial	Final	
1*	2.38	0.26	0	2.09	0	2.00	62.5
2†	0	2.64	9.55	6.60	9.55	6.60	58.9
3‡	2.38	0.24	0	2.10	0	2.10	52.0
4§	2.38	0.25	0	2.14	0	2.14	51.0

* Reaction components and conditions: 10 μ moles of DL-allothreonine, 0.1 μ mole of PLP, 30 mg of enzyme protein, 10^{-3} M phosphate buffer, to give 2.1 ml per vial. Incubation time, 2.5 hours.

† Reaction components and conditions were the same as those in Experiment 1, except for 20 μ moles of acetaldehyde and 20 μ moles of glycine in place of DL allothreonine.

‡ Reaction components and conditions were the same as those in Experiment 1, except for 60 mg of enzyme protein.

§ Reaction components and conditions were the same as those in Experiment 1, except for 90 mg of enzyme protein.

Reversibility and Equilibrium of Reaction

The synthesis of threonine from acetaldehyde and glycine was shown to occur non-enzymatically by Metzler *et al.* (13) and has been reported to take place enzymatically by Gilbert (4). The reversibility of the reaction was verified by us, and experiments were performed to determine the equilibrium constant defined as

$$K_{eq} = \frac{(\text{allothreonine})}{(\text{acetaldehyde})(\text{glycine})}$$

In these experiments it was assumed that the increase in acetaldehyde was equivalent to the decrease in allothreonine concentration in the forward reaction. This assumption is supported by the stoichiometry of the reaction (Table V).

In the reverse reaction, allothreonine formation was assumed to equal the quantity of material which reacted with periodate minus the amount of threonine present, as determined by biological assay. This assumption was checked by quantitative paper chromatography, with a solvent mixture of butanol, water, acetone, and ammonia (1 0 75 0 12 0 12) (14). The data are reported in Table VI. K_{eq} values of 51 0, 52 0, and 58 9 were calculated for three different concentrations of enzyme at pH 7 6 for the forward reaction. With the reverse reaction a value of 62 5 was obtained. It is possible that the final equilibrium position was not quite achieved in either direction, and that the best value of the equilibrium constant is an average of the above figures, about 56.

Calculation of the equilibrium constant for threonine was unsatisfactory, giving no agreement in the figures for the reaction in the forward and reverse direction.

DISCUSSION

The evidence reported in this work suggests that two separate enzymes are involved in both the decomposition and synthesis of threonine and allothreonine, although it has not been possible up to now to separate completely the two enzyme activities. This evidence consists of the variation in the activity ratio on the two substrates upon fractionation and differences in the response under various experimental conditions.

A possible mechanism for threonine and allothreonine breakdown which involved the participation of pyridoxal and metal ions, *viz.* iron, aluminum, or chromium, was presented by Metzler *et al.* (13). The threonine aldolases are activated by PLP⁶. However, no metal ion or other organic cofactor requirement could be shown, and no inhibition could be produced by chelating agents. The negative results with metal ions and sequestering agents cannot rule out the possibility that a metal ion may be a cofactor for crystalline aldolases, but, at the present time, an earlier mechanism of reaction (15) which does not involve the participation of a metal ion can explain the experimental findings.

The presence of allothreonine aldolase cannot be readily explained by the existing data for this amino acid. Allothreonine is not a naturally occurring amino acid,⁶ it cannot prevent the formation of fatty livers on threonine-deficient diets (17), and is a compound for which no biochemical significance has been adduced. This poses the possibility that allothreonine is not the natural substrate for this enzyme and that its breakdown is merely an artifact.

⁶ Tests performed in our laboratory to demonstrate the incorporation of DL-allothreonine-1,2-C¹⁴ into protein of the body tissues, and a survey of some naturally occurring plant and animal proteins for the presence of allothreonine, yielded completely negative results (for experimental details, see Karasek (16)).

The implications of the reversibility of threonine breakdown for metabolism deserve comment. Threonine can be synthesized *in vitro* from acetaldehyde and glycine by enzymes from rat and sheep liver. Also, the magnitude of the equilibrium constant favors the synthetic reaction (for allothreonine), although not as strongly as was found for the corresponding reaction for the biosynthesis of serine from formaldehyde and glycine (11), *viz* $K_{eq} = 2.76 \times 10^3$, as against 56.

The reason for the indispensability of threonine in the diet of mammals may possibly be the lack of a known metabolic pathway that can produce sufficient quantities of acetaldehyde to favor a rate of synthesis of threonine adequate to meet the nutritional requirement.⁷ It should be noted, however, that, in spite of the favorable equilibrium constant for synthesis, at concentrations normal for biological material the decomposition of the threonines rather than synthesis would be greatly favored. Meltzer and Sprinson (18), from a study of the fates of L-threonine labeled with C¹⁴ and N¹⁵, concluded that one-fifth to one-third of dietary threonine is cleaved in the rat to glycine. From this result it has been inferred that the major pathway of threonine dissimilation is by way of the threonine aldolase reaction. The weak activity of threonine decomposition and the high activity of threonine dehydrase (19) found in liver make this a dubious conclusion. In addition, it has been found that threonine dehydrase is inducible and is increased in activity by threonine administration (20), while the threonine aldolases are not.

SUMMARY

1 Evidence has been secured for the presence of two distinct enzymes in sheep liver that cleave threonine and allothreonine to glycine and acetaldehyde. The rate of decomposition of threonine in crude preparations is one-twentieth of that of allothreonine. The specificity of each reaction is confirmed by the differences in the effects on the two substrates of enzyme concentration, incubation time, pyridoxal phosphate activation, inhibition by chemical analogues, and ammonium sulfate fractionation.

2 The only cofactor requirement detected was for pyridoxal phosphate. Other vitamin B₆ derivatives were ineffective. No requirement could be demonstrated for metal ions or other organic cofactors.

3 Evidence was secured for the reversibility of both reactions, and the equilibrium constant for the cleavage of allothreonine was measured.

4 The general properties of threonine and allothreonine aldolases were

⁷ An attempt to replace or reduce the dietary requirement for threonine by administering drinking water containing 0.5 per cent glycine and 10 per cent ethanol on a threonine-deficient diet failed to show any substitution for threonine, as evidenced by the weight response (for experimental details, see Karasek (16)).

studied. The pH optima, effect of ionic strength, and stability were similar for both. The enzyme-substrate dissociation constants of pyridoxal phosphate for the two enzyme activities and of threonine and allothreonine have been determined.

5. Experiments with *p*-chloromercuribenzoate, iodoacetate, *O*-iodosobenzoate, glutathione, cysteine, and dimercaptopropanol showed that both enzymes required free —SH groups for enzyme activity.

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CORTICOTROPINS

XIII IDENTIFICATION OF CERTAIN PEPTIDES IN PARTIAL ACID HYDROLYSATES OF α -CORTICOTROPIN (α -ACTH)*

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The complete amino acid sequence of α -corticotropin (α -ACTH) has recently been reported (1). In order to establish this sequence, investigation of peptide fragments obtained by partial acid hydrolysis of the dimethylphenyl (DNP) derivative of the peptide hormone had been undertaken. In addition, in order to confirm the sequence of certain of the amino acids, peptide fragments derived from partial acid hydrolysis of α -ACTH itself were also recently investigated. Such studies were felt to be of particular importance in order to ascertain whether transpeptidation near the basic amino acids had occurred during the enzymatic digestion in the course of the structural investigation (1).

EXPERIMENTAL

Analysis of Peptides Obtained from DNP α -ACTH—40 mg of DNP α -ACTH (2) were suspended in 0.5 ml of constant boiling HCl, in a test tube subsequently sealed under a vacuum, and were maintained at 110° for 5 hours, until dissolution was complete. The DNP peptides were then removed from the hydrolysate by successive extractions three times with diethyl ether, three times with ethyl acetate, and once with *n*-butanol. Each of these extracts, and the remaining aqueous phase, was evaporated to dryness and submitted to paper chromatography in the solvent system *tert*-amyl alcohol-isoamyl alcohol-3 per cent NH_4OH (2:1:3) (see Fig. 1).

Spots 1 to 6 from the butanol extract (Fig. 1, B) and Spots 1 to 8 from the aqueous phase (Fig. 1, W) were cut from the paper, and the compounds were eluted with 3 per cent NH_4OH . The eluates were then chromatographed on paper with a phosphate buffer (1.6 M, pH 7) as the developer (Figs. 2 and 3). In most instances, single spots from amyl alcohol chromatograms

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could be resolved into two components (1 and 1a, 2 and 2a, etc), which were cut out separately and eluted. The compounds in each of the eluates were dinitrophenylated in order to substitute the α -amino group, acidified to pH 1, and again fractionated by extraction in the same way with diethyl ether, ethyl acetate, and *n*-butanol. The dinitrophenylation is usually carried out as follows. The peptides are dissolved in 0.5 ml of 5 per cent

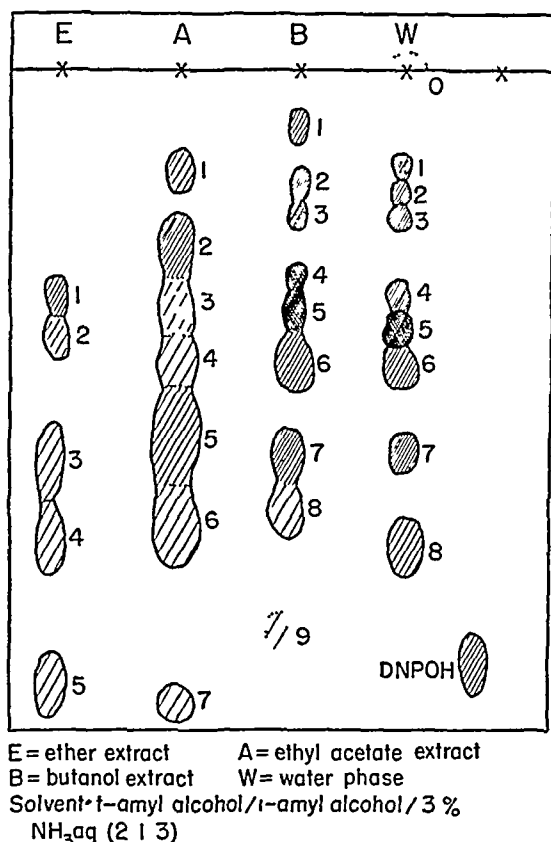


FIG 1 Paper chromatogram of extracts from partial acid hydrolysate of DNP α -corticotropin

NaHCO_3 , and 10 ml of a 2 per cent solution of dinitrofluorobenzene (DNFB) in EtOH (v/v) is added. The mixture is shaken at room temperature for 3 hours (4 hours for amino acid analyses) and is then diluted with 2 volumes of water and extracted three times with diethyl ether to remove excess DNFB and other side products of the reaction, after acidification of the resulting solution with 2 drops of concentrated HCl, extraction (three times) is performed with diethyl ether.

The material obtained from the origin of the chromatogram of the aqueous phase (Spot 0, Fig 1) contained most of the peptides from the

original hydrolysate that were not dinitrophenylated. This mixture was then submitted to dinitrophenylation and fractionated by extraction as described above, and was finally separated by chromatography in a two-dimensional system (3). The complexity of this fraction is illustrated in Fig 4. The materials obtained in this manner, as well as the other DNP peptide fractions mentioned above, were submitted to complete hydrol-

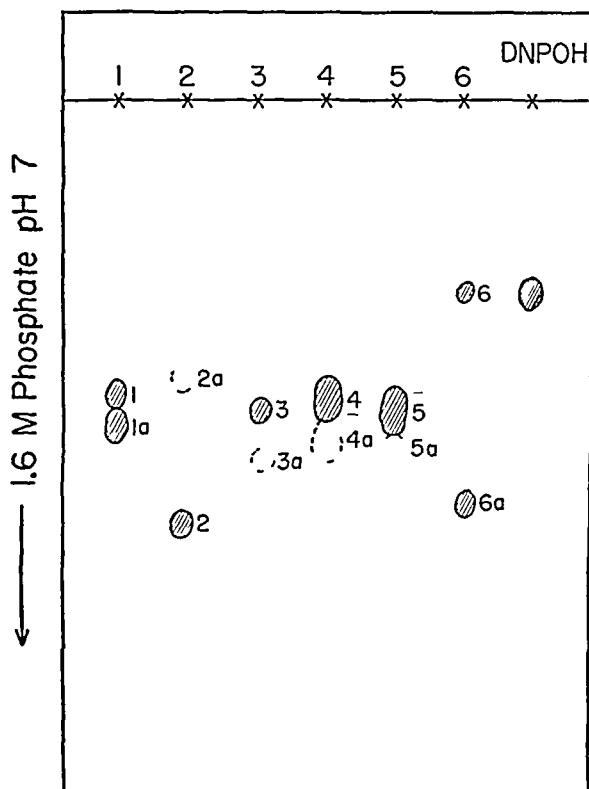


Fig 2 Paper chromatogram of DNP peptides in the butanol extract, previously separated by paper chromatography (see Fig 1). DNPOH, dinitrophenol.

ysis (with constant boiling HCl, at 110° for 16 to 30 hours, in tubes sealed under a vacuum), followed by extraction with diethyl ether. The DNP amino acids thus extracted, which represent *N*-terminal residues of the corresponding peptides, were identified by means of paper chromatography (3). The aqueous phase remaining after the extraction contained free amino acids, *O*-DNP tyrosine and mono-DNP derivatives of basic amino acids. These were identified on paper by using the butanol-acetic acid-water (4:1:5) system (4), the amino acid concentration was estimated from the intensity of ninhydrin color of each spot of the chromatogram. When a single α -DNP amino acid was obtained from a hydrolysate of

peptide material, it was assumed that the hydrolysate was derived from a single peptide

Analysis of Peptides Obtained from α -Corticotropin—25 mg of α -ACTH (5) were dissolved in 0.5 ml of concentrated HCl and incubated at 40° for 4 days. The solution was then evaporated three times to dryness, *in vacuo* over NaOH, in order to remove all excess HCl. Finally, the residue was redissolved in 0.5 ml of H₂O, and the solution was applied to a

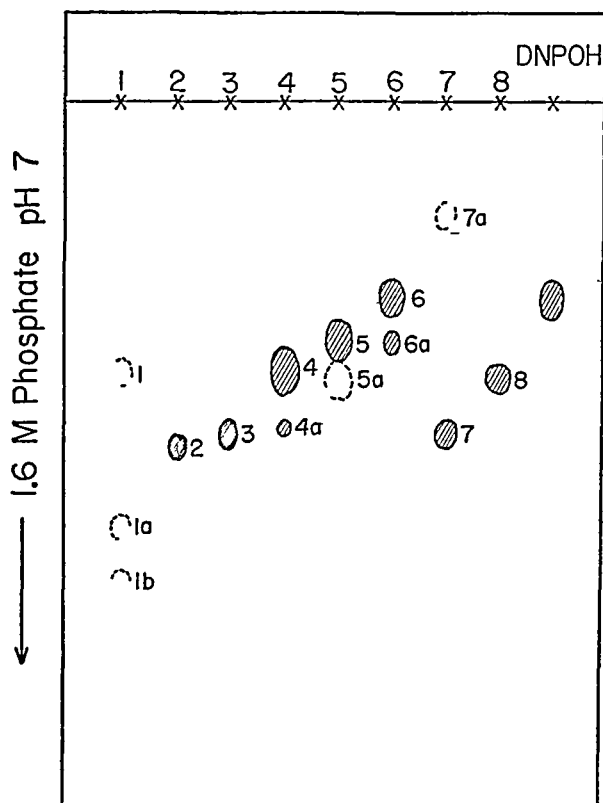


FIG 3 Paper chromatogram of DNP peptides in the aqueous phase, previously separated by paper chromatography (see Fig 1)

large sheet of Whatman No 3 MM filter paper for electrophoretic separation in a Spinco apparatus (6) at 200 volts for 7 hours in pH 6.5, collidine-acetic acid buffer (7) at room temperature. After the paper was dried, two narrow strips were excised for development with ninhydrin and for detection of arginine by the Sakaguchi reaction (8). Fig 5 shows the ninhydrin pattern from the origin to the cathode, a solid dot indicates the presence of arginine.

With this pattern as a guide, the remainder of the sheet was cut, and the strips were eluted with 3 per cent NH₄OH. The solvent was evaporated and the residues were then redissolved in water. An aliquot from each of

these solutions
Whatman No. 1
sisting of



FIG 4 Paper chromatography of peptides in Spot W-6

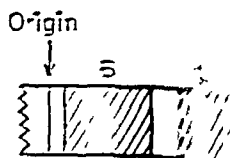
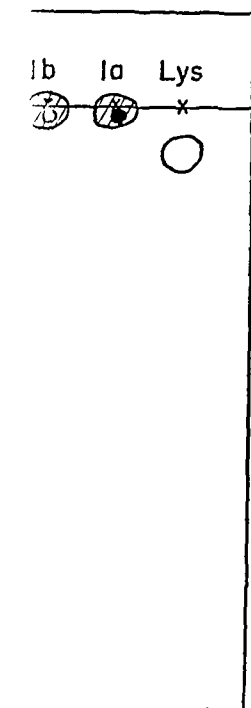


FIG 5 Zone electrophoresis of tropin, solvent *n*-butanol, 100 volts for 7 hours. The effect of the Sakaguchi reaction,

(9) Fig 6 shows the results of the reactions. Each of the three strips across a large plate of acetic acid-pyridine was developed and eluted with 3 per cent in the system *n*-butanol



spots shown in Fig 6

Partial Acid Hydrolysis of Papain by Paper Electrophoresis

idst	Probable sequence
	Lys Arg
	Arg Arg
y)	Phe(Arg, Try)
g, Arg	Lys(Lys, Arg, Arg)
	Arg Arg
	Phe Arg
	Arg Pro
y, Val	Lys(Pro, Val)
al	Lys Val
none	Lys
"	Arg

phase retraction hydroly-
applied DNP tanol-
hydr the

peptide material, it was assumed that the hydrolysate was derived from a single peptide

Analysis of Peptides Obtained from α -Corticotropin—25 mg of α -ACTH (5) were dissolved in 0.5 ml of concentrated HCl and incubated at 40° for 4 days. The solution was then evaporated three times to dryness, *in vacuo* over NaOH, in order to remove all excess HCl. Finally, the residue was redissolved in 0.5 ml of H₂O, and the solution was applied to a

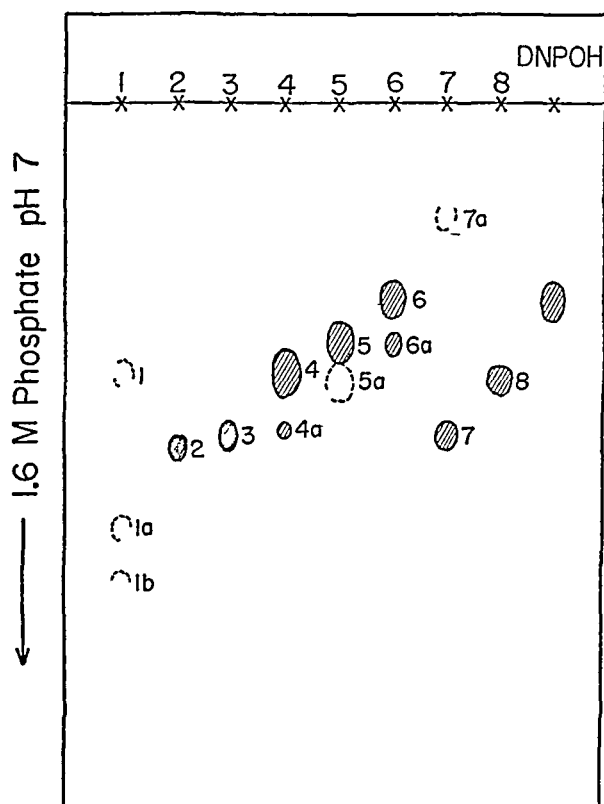


FIG 3 Paper chromatogram of DNP peptides in the aqueous phase, previously separated by paper chromatography (see Fig 1)

large sheet of Whatman No. 3 MM filter paper for electrophoretic separation in a Spinco apparatus (6) at 200 volts for 7 hours in pH 6.5, collidine-acetic acid buffer (7) at room temperature. After the paper was dried, two narrow strips were excised for development with ninhydrin and for detection of arginine by the Sakaguchi reaction (8). Fig 5 shows the ninhydrin pattern from the origin to the cathode, a solid dot indicates the presence of arginine.

With this pattern as a guide, the remainder of the sheet was cut, and the strips were eluted with 3 per cent NH₄OH. The solvent was evaporated and the residues were then redissolved in water. An aliquot from each of

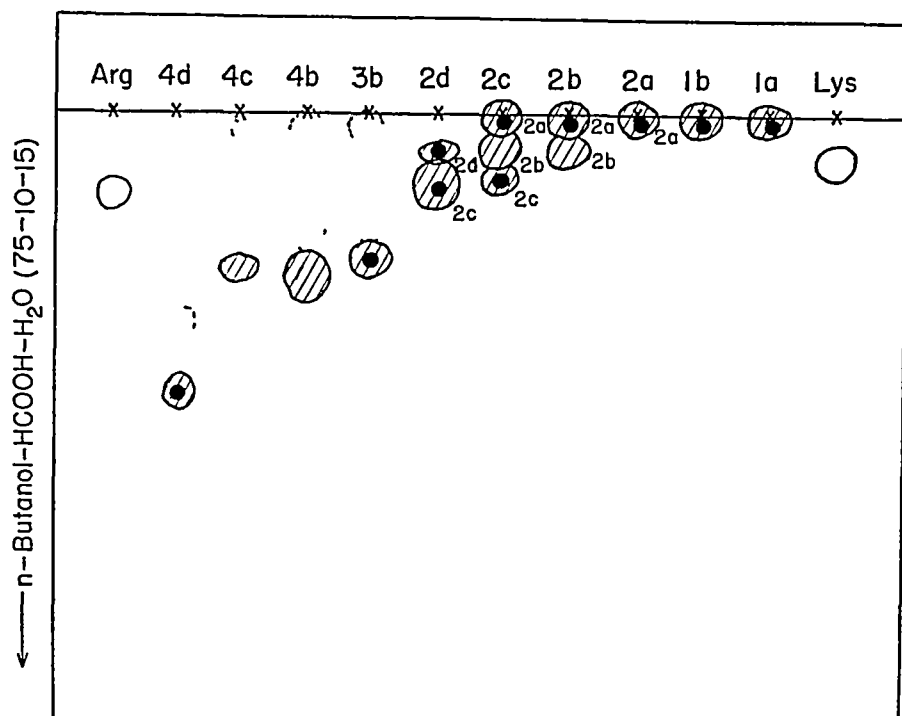


FIG 7 Paper chromatogram of fractions eluted from the spots shown in Fig 6

TABLE I

Analysis of Some Peptides from Paper Chromatograms of Partial Acid Hydrolysis of α -Corticotropin after Preliminary Separation by Paper Electrophoresis

Experiment No	Peptide No *	N Terminal residue	Residual amino acids†	Probable sequence
I	1b	Lys	Arg	Lys Arg
	1, 2a	Arg	"	Arg Arg
	4b	Phe	Arg, (Try)	Phe(Arg, Try)
II	1a	Lys	Lys, Arg, Arg	Lys(Lys, Arg, Arg)
	2a	Arg	Arg	Arg Arg
	2d	Phe	"	Phe Arg
	3b	Arg	Pro	Arg Pro
	4b	Lys	Pro, Val	Lys(Pro, Val)
	4c	"	Val	Lys Val
	2b	"	None	Lys
	2c	Arg	"	Arg

* See Fig 7

† Amino acid present in the aqueous phase after ether extraction of the acid hydrolysis

did not give a yellow-colored ether extract was applied to the *n*-butanol-formic acid-water system for the estimation of DNP arginine, since the latter is not ether-soluble. The aqueous acid hydrolysates were then

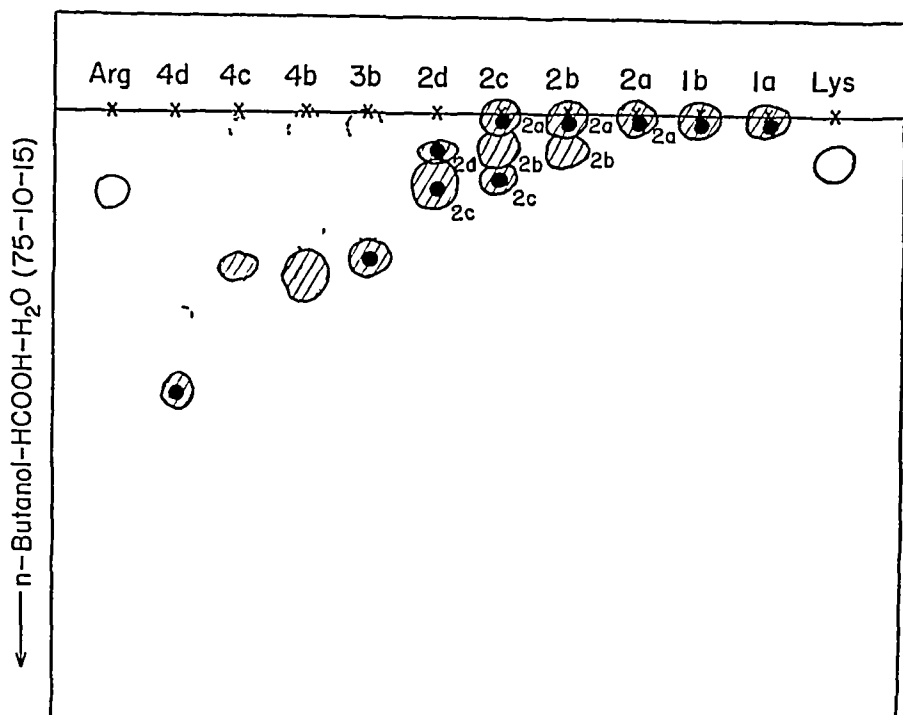


Fig 7 Paper chromatogram of fractions eluted from the spots shown in Fig 6

TABLE I

Analysis of Some Peptides from Paper Chromatograms of Partial Acid Hydrolysis of α -Corticotropin after Preliminary Separation by Paper Electrophoresis

Experiment No	Peptide No *	N Terminal residue	Residual amino acids†	Probable sequence
I	1b	Lys	Arg	Lys Arg
	1,2a	Arg	"	Arg Arg
	4b	Phe	Arg, (Try)	Phe(Arg, Try)
II	1a	Lys	Lys, Arg, Arg	Lys(Lys, Arg, Arg)
	2a	Arg	Arg	Arg Arg
	2d	Phe	"	Phe Arg
	3b	Arg	Pro	Arg Pro
	4b	Lys	Pro, Val	Lys(Pro, Val)
	4c	"	Val	Lys Val
	2b	"	None	Lys
	2c	Arg	"	Arg

* See Fig 7

† Amino acid present in the aqueous phase after ether extraction of the acid hydrolysis

did not give a yellow-colored ether extract was applied to the *n*-butanol-formic acid-water system for the estimation of DNP arginine, since the latter is not ether-soluble. The aqueous acid hydrolysates were then

evaporated and again dinitrophenylated for the quantitative estimation of the amino acid composition of the peptide (minus the *N*-terminal amino acid)

Results

DNP α -Corticotropin—Most spots in the paper chromatograms (Figs 1 to 4) obtained from the partial acid hydrolysate of DNP α -ACTH contained either mixtures of peptides or peptides which were too complex to yield a conclusive identification, nine of these spots, however, seemed to

TABLE II
Analysis of Some DNP Peptides Obtained from Partial Acid Hydrolysis of DNP α -Corticotropin

Peptide No	<i>N</i> -Terminal residue	Residual amino acids*	Probable sequence
B-2†	di-DNP-Lys	Pro, Val, Gly	Lys(Pro, Val, Gly)
B-3†	“	Val, Tyr, Pro	Lys(Val, Tyr, Pro)
B-4†	DNPOH‡	Pro, Val, ϵ -DNP-Lys	Pro(Val, Lys)
B-5†	“ ‡	Pro, Val ₂ , Tyr, ϵ -DNP-Lys	Pro(Val, Lys, Val, Tyr)
W-01§		DNP-Arg, Pro	Arg Pro
W-03§	DNPOH‡	(ϵ)DNP-Lys, Pro, Val, Gly	Gly(Lys, Pro, Val, Gly)
W-04§	DNP-Ala	Gly, Glu, Asp	Ala(Gly, Glu, Asp)
W-2	di-DNP-Lys	Arg	Lys Arg
W-3	“	Val	Lys Val

* Amino acid present in the aqueous phase after ether extraction of the acid hydrolysate of the DNP peptide

† See Fig 2

‡ DNP proline and DNP glycine are known to decompose under the conditions of hydrolysis to give rise to dinitrophenol (DNPOH)

§ See Fig 4

|| See Fig 3

arise from single peptides whose structure could be deduced (Table II). Among these peptides, W-04 (Table II) is the only one containing neither arginine nor lysine and probably represents the tetrapeptide occupying positions 25 to 28 in the α -ACTH molecule (1).

Since one of the two tyrosine residues known to occur in the α -ACTH molecule is located at the *N*-terminus in the sequence Ser Tyr Ser, etc (10), and since from their amino acid composition it is evident that peptides B-3 and B-5 (Table II) are not derived from the *N*-terminus, both the tyrosines found in these peptides must represent the second tyrosine residue. Thus, it is possible to assume the existence of the sequence Pro Val Lys Val(Tyr, P₁₀). Finally, the sequence Gly Lys(Pro, Val, Gly) may be formulated from the probable sequence of the peptides B-2 and W-03.

α -Corticotropin—Two separate experiments with two different preparations of α -corticotropin were carried out. Although the conditions used for hydrolysis were the same, the patterns obtained were not identical with respect to the nature and the amount of each peptide. This is to be expected, as it is well known that partial acid hydrolysis of proteins or peptides is far from being a reaction with high specificity.

The peptides which were investigated were those giving strong ninhydrin and Sakaguchi reactions. Most of them were found to be homogeneous after paper chromatography, Table I shows the results of these analyses. It should be noted that peptides 2d of Experiment II and 4b of Experiment I (Table I) were identical as far as the results of the amino acid analyses were concerned. However, because of the difference in their mobilities in paper electrophoresis and of their R_F values in paper chromatography, it was concluded that 4b (Experiment I) is more "fatty" and less "basic" than 2d (Experiment II). From evidence previously reported (1), it appears that peptide 2d (Experiment II) is Phe Arg while 4b (Experiment I) is Phe(Arg,Try), the tryptophan being destroyed during the acid hydrolysis and consequently not appearing in the analysis. Since from chymotryptic digests of α -ACTH the sequence Arg Try was identified (1), and since only 1 tryptophan residue occurs in the molecule (11), the sequence Phe Arg Try may therefore be assumed to occur in the peptide hormone. From peptides 1b and 2a (Experiment I) and 2a and 1a, Experiment II, it is evident that a sequence, Lys Lys Arg Arg, can be established.

SUMMARY

Peptides containing lysine and arginine derived from the partial acid hydrolysis of α -corticotropin and dimethylphenyl α -corticotropin have been isolated by chromatographic and electrophoretic procedures. The following sequences have been identified: Lys Arg, Arg Phe, Pro Val Lys - Val(Tyr, Phe), Gly Lys(Phe, Val, Gly), Ala(Gly, Glu, Asp), Lys Val, Lys - (Pro, Val), Phe Arg Try, and Lys Lys Arg Arg. Since these sequences are consistent with the proposed amino acid sequence for α -corticotropin (1) as derived from enzymatic digests, it seems unlikely that transpeptidation by the enzymes employed for hydrolysis of the peptide hormone has occurred.

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CHARACTERISTICS OF THE INHIBITION BY ETHIONINE OF THE INCORPORATION OF METHIONINE INTO PROTEINS OF THE EHRlich ASCITES CARCINOMA IN VITRO*

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(Received for publication, January 14, 1957)

The principal characteristic of an antimetabolite is its ability to block the normal functions of the corresponding natural metabolite. The toxicity of several antimetabolites, however, appears to be due to a product which arises only after the antimetabolite has been transformed by enzymatic reactions which are normally reserved for the natural metabolite (2). In support of this concept, Levine and Tarver (3) demonstrated that labeled ethionine is incorporated into the mixed proteins of rat tissues, and suggested that its toxicity may in part be due to the newly formed unnatural proteins. Subsequently, peptides containing ethionine (4) and *p*-fluorophenylalanine (5) have been isolated from proteins of *Tetrahymena pyriformis* and *Lactobacillus arabinosus* after these organisms had been grown in media that contain the respective analogues. More recently, reports have appeared that *Escherichia coli* can synthesize proteins containing either azatryptophan (6) or *p*-fluorophenylalanine (7).

The observation that amino acid analogues may become incorporated into protein made it desirable to determine some quantitative characteristics of this process. This was of particular interest since ethionine did not block the incorporation of radioactive leucine into the protein of rat liver microsomes (8), nor did phenylalanine analogues prevent the incorporation of several amino acids into the protein of Ehrlich ascites cells (9). If amino acid analogues can enter protein by substituting for their natural metabolite, it might be possible to explain their failure to inhibit amino acid incorporation into proteins, particularly in cases in which only trace levels of such protein may be formed. This communication describes the relationship between methionine and ethionine in the protein-synthetic mechanism of Ehrlich ascites cells under *in vitro* conditions.

* Aided by grants from the Damon Runyon Memorial Fund for Cancer Research, Inc., and the Cancer Research Funds of the University of California. A preliminary report of this work has been presented (1).

EXPERIMENTAL

Methods

The radioactive amino acids other than methionine and ethionine were previously described (9) L-Methionine-methyl- C^{14} , 5.6 μ c per mg, was obtained from the Isotopes Specialties Company, Inc., Burbank, California. The L-ethionine-1-ethyl- C^{14} , 7.4 μ c per mg, was kindly provided by Dr. Gross and Dr. Tauber, who have described its preparation (4). Non-radioactive L-ethionine and L-methionine, which were mixed with their radioactive counterparts to obtain the required specific activities, were purchased from the California Foundation for Biochemical Research, Los Angeles.

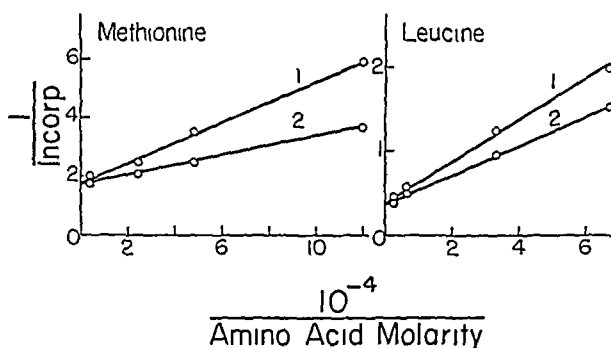


FIG. 1. Double reciprocal plot showing competitive inhibition by ethionine of methionine and leucine incorporation into Ehrlich ascites cell protein. Curves 1, inhibition by L-ethionine, 5×10^{-3} M; Curves 2, uninhibited incorporation. Incorporation is expressed as micromoles of amino acid incorporated per gm. of protein during the 15 minute incubation period.

Incubations, protein isolation, and counting procedures were carried out as previously described (9). In addition, all protein samples containing labeled methionine or ethionine were dissolved in formic acid and oxidized by the addition of hydrogen peroxide (9). The preparations of the proteins obtained in this manner had constant specific activity upon repetition of this process.

Characteristics of Inhibition by Ethionine—The incorporation of radioactive methionine into Ehrlich ascites cell protein was inhibited by ethionine. As shown in Fig. 1, this inhibition was competitive. Ethionine also inhibited the incorporation of other amino acids. However, as in the case of phenylalanine antagonists (9), this inhibition could be relieved by merely increasing the concentration of the amino acid being incorporated. This competitive inhibition by ethionine of the incorporation of amino acids other than methionine is also illustrated in Fig. 1 by its effect on leucine incorporation. If incorporation of radioactive amino acids reflects

protein synthesis, and if ethionine blocks this process, one would anticipate a *non-competitive* inhibition of incorporation of amino acids other than methionine. The following observations support the concept that ethionine does not inhibit protein synthesis *in vitro* in this cell system, but by displacing methionine participates in the formation of an unnatural protein.

Energy Requirements for Ethionine Incorporation—Ethionine is incorporated into Ehrlich ascites cell protein. Table I shows that this occurred under anaerobic conditions only when supported by active glycolysis. Also, under aerobic conditions, ethionine incorporation was inhibited by low concentrations of dinitrophenol. These data indicate that, as in the case of the natural amino acids (10), the incorporation of ethionine requires a source of energy.

TABLE I

Energy Requirement for Ethionine Incorporation into Ehrlich Ascites Cell Protein

Aerobic		Anaerobic	
Uninhibited	With dinitrophenol, 5×10^{-6} M	Glucose, 0.015 M	Without glucose
$0.70 \pm 0.00^*$	0.40 ± 0.01	0.365 ± 0.005	0.027 ± 0.004

* Incorporation is expressed as micromoles of ethionine incorporated per gm. of protein during the 1 hour incubation \pm deviation from the mean of duplicate incubations. The L-ethionine concentration was 5×10^{-3} M.

Ethionine, Competitive Substrate for Methionine—Ethionine was effectively incorporated into Ehrlich ascites cell protein only when it was present in concentrations high enough to inhibit methionine incorporation. Unlike methionine, the incorporation of ethionine was markedly dependent upon its concentration (Fig. 2). The calculated ratio of the apparent Michaelis-Menten constants for the incorporation of methionine and ethionine indicated that the *affinity* of the Ehrlich ascites cell for incorporation of methionine was 600 times that for ethionine. In contrast to the marked difference in affinities, the identity of the intercepts on the reciprocal incorporation axis (Fig. 2) may be interpreted as indicating that the *capacity* of the protein-forming systems for the incorporation of these two amino acids was identical. This equivalence of incorporative capacities implies that the same sites are involved in both methionine and ethionine incorporation.

The above conclusion was confirmed by a balance study in which the inhibition of methionine incorporation and the concurrent incorporation of ethionine were compared. This balance is shown for three methionine

concentrations in Table II. The data in line 2 of this table show that, in the presence of very low concentrations of added methionine, more ethionine was incorporated (0.25 μ mole) than could be accounted for by the corresponding loss in methionine incorporation (0.11 μ mole)

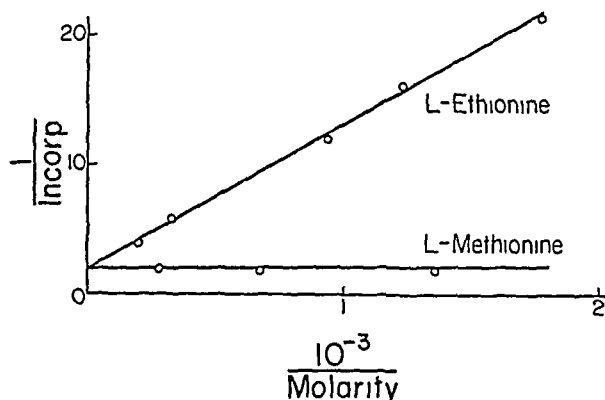


FIG. 2. The effect of concentration of radioactive L-methionine and L-ethionine upon their incorporation into Ehrlich ascites cell protein. Incorporation is expressed as in Fig. 1.

TABLE II
Competition between Methionine and Ethionine for Incorporation into Protein of Ehrlich Ascites Cells

L. Methionine M	Incorporation, μ mole per gm. protein per 15 min. incubation period		
	L. Methionine C^{14}		L. Ethionine C^{14} , $5 \times 10^{-3} M$
	Alone	With L. ethionine C^{12} , $5 \times 10^{-3} M$	
0.0 $\times 10^{-3}$			0.31
0.0082 $\times 10^{-3}$	0.27	0.16	0.25
0.0205 $\times 10^{-3}$	0.45	0.28	0.20
0.123 $\times 10^{-3}$	0.57	0.47	0.08

The difference may be due to the incorporation of endogenous cellular methionine. A stoichiometric relationship between ethionine incorporation and loss in methionine incorporation was obtained when the higher concentrations of methionine were used.

Characteristics of Incorporated Ethionine—Incorporated ethionine is distributed between the buffer-soluble proteins and the proteins of the cell particles to about the same extent as methionine (Table III). No evi-

dence could be found for the accumulation of ethionine in the particle fraction of the Ehrlich ascites cell, which contains the ribonucleoprotein reported to be engaged in the preliminary step of amino acid incorporation (11). This may be taken as an indication that ethionine is rapidly incorporated into the completed protein. Furthermore, as shown in Fig 3, only a small fraction of incorporated ethionine (about 10 per cent) is released from protein in cells which are actively incorporating methionine. In view of the much greater affinity of the cells for the natural substrate and the prolonged time of incubation with methionine, one might anticipate that exchangeable ethionine would be more readily displaced. The

TABLE III
*Distribution of Incorporated Methionine and Ethionine between
Particulate and Soluble Proteins of Ehrlich Ascites Cells*

Incorporation, μ mole per gm protein			
L-Methionine, $0.0082 \times 10^{-3} M$		L-Ethionine, $5 \times 10^{-3} M$	
Soluble protein	Particulate protein	Soluble protein	Particulate protein
$0.19 \pm 0.006^*$	0.18 ± 0.009	0.27 ± 0.015	0.26 ± 0.003

* Standard error of the mean of triplicate incubations. The concentrations of methionine and ethionine were adjusted to give the same order of incorporation during the 15 minute incubation period. After the incubation, the cells, together with their medium, were cooled in ice and then exposed for 3 to 4 minutes in a Raytheon 9 kc magnetostriction oscillator at full power. The particulate matter was then centrifuged at $100,000 \times g$ for 90 minutes.

released ethionine may arise from the breakdown of unnatural protein within the cell (3, 12).

Ethionine Incorporation As Aberrant Protein Synthesis—We have previously reported that the incorporation of some natural amino acids is inhibited by analogues of other amino acids and that the inhibition can be prevented only by the presence of the corresponding metabolite (13). The incorporation of ethionine was also inhibited by such analogues. As shown in Table IV, *O*-methylthreonine (14) and δ -hydroxylysine (15) partially inhibited the incorporation of ethionine, this inhibition could be prevented by isoleucine and glutamine, respectively. Methionine sulfoximine, which, like δ -hydroxylysine, inhibits protein synthesis by blocking glutamine synthesis (16), was not an effective inhibitor of ethionine incorporation. This may be due to prevention by ethionine of the absorption of methionine sulfoximine by the cell, a phenomenon common among amino acids of similar structure (17).

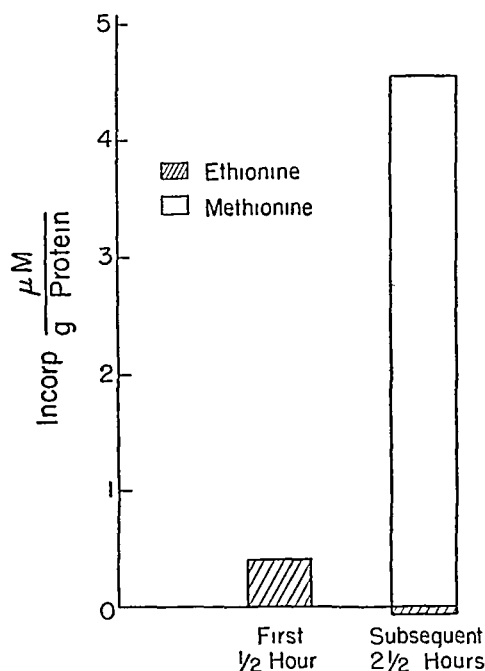


FIG 3 An attempt to displace ethionine incorporated into Ehrlich ascites cell protein by methionine Ehrlich ascites cells were incubated with 10 μ moles of L-ethionine in 2 ml of buffer for 30 minutes, then 10 μ moles of L-methionine in 0.2 ml were added When radioactive, this methionine was incorporated during the subsequent 2.5 hours as shown When the methionine was not radioactive, the amount of ethionine released is shown beneath the bar for methionine 20 μ moles of sodium pyruvate were present in all flasks to support respiration during the prolonged incubation

TABLE IV

Inhibition of Ethionine Incorporation into Ehrlich Ascites Cell Protein by O-Methylthreonine and δ -Hydroxylysine and Its Prevention by Isoleucine and Glutamine, Respectively

Incorporation of L-ethionine, μ mole per gm protein per 15 min incubation period					
Uninhibited control	With O-methylthreonine	With O-methylthreonine and isoleucine	With δ -hydroxylysine	With δ -hydroxylysine and glutamine	With glutamine
0.23 \pm 0.005*	0.17 \pm 0.004	0.20 \pm 0.009	0.19 \pm 0.01	0.26 \pm 0.004	0.26 \pm 0.009

* Deviation from the mean of duplicate determinations The concentrations were L-ethionine, 5×10^{-3} M, O-methyl-DL-threonine, 2×10^{-2} M, L-isoleucine, 5×10^{-4} M, δ -hydroxy-DL-lysine, 2×10^{-3} M, L-glutamine, 5×10^{-4} M

DISCUSSION

The behavior of amino acid antagonists as competitive substrates appears to be the most common action of such analogues on the amino acid-incorporating system of Ehrlich ascites cells This effect explains the

previously reported independent antagonism of phenylalanine by *o*-fluorophenylalanine and β -2-thienylalanine (9). Many other amino acid analogues also inhibit only the incorporation of their respective metabolites without blocking the incorporation of other amino acids¹. Two other types of antagonism have also been found, the direct inhibition of protein synthesis by *O*-methylthionine, an isoleucine antagonist (14), and the indirect inhibition of synthesis, through the prevention of formation of glutamine, an essential amino acid in the Ehrlich ascites cell (16).

After observing that radioactive ethionine is incorporated into proteins of the rat, Levine and Tauber (3) speculated that some of the metabolic effects of ethionine may be ascribed to the toxicity of the unnatural proteins formed. The work reported here, together with similar studies on microorganisms (4-6), supports the probability of their conclusion. However, the analogues may enter only the more non-discriminating proteins and block the formation of those which will not accept minor structural modifications. This appears to be the case in growth studies with *L. arabinosus*, in which *p*-fluorophenylalanine will support growth only when the medium contains suboptimal levels of phenylalanine (5). This point is also supported by the observations of Paidee, Shore, and Priestidge, who reported (6) that some enzymes may be formed in the presence of azatryptophan whereas others are formed either in an inactive state or not at all.

The low affinity for the incorporation of ethionine by the Ehrlich ascites cell ($\frac{1}{800}$ that of methionine) and the resulting higher concentrations necessary to support incorporation of the unnatural amino acid indicate a structural specificity for protein formation which is much higher than that usually observed for enzymes which act on amino acids (18) or their derivatives (19).

The high concentration of ethionine required to demonstrate a competitive substrate relationship, together with the fact that only the initial rates of incorporation were observed in these *in vitro* studies, suggests that any extension of these results to the action of ethionine on protein metabolism in the intact animal should be made with caution.

SUMMARY

1 Ethionine inhibits the *in vitro* incorporation of methionine into proteins of the Ehrlich ascites cell.

2 The antagonist is itself incorporated, and, as in the case of natural amino acids, this incorporation requires an energy source.

3 All inhibition of incorporation of methionine can be ascribed to the incorporation of ethionine, which acts as a competitive substrate.

4 The affinity for incorporation of ethionine, however, is only $\frac{1}{800}$ that

¹ Rabinovitz, M., Olson, M. E., and Greenberg, D. M., unpublished observations.

for methionine Once ethionine is incorporated, it is not readily released by methionine

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SOME CHARACTERISTICS OF RAT KIDNEY 5-HYDROXYTRYPTOPHAN DECARBOXYLASE[†]

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(Received for publication, January 11, 1957)

Several reports have appeared which describe the occurrence and properties of 5-hydroxytryptophan decarboxylase in guinea pig and hog kidney (1-3)

Although a pyridoxal phosphate requirement has been demonstrated for a number of amino acid decarboxylases (4-7) and it has been indicated that such a requirement may exist for 5-hydroxytryptophan decarboxylase (1, 3, 8), conclusive demonstration of this relationship for the latter enzyme has not been made. The results presented here show a pyridoxal phosphate requirement and describe several other characteristics for this reaction in rat kidney homogenates.

EXPERIMENTAL

Materials—5-Hydroxy-DL-tryptophan, 5-hydroxytryptamine creatinine sulfate, pyridoxal hydrochloride, thiamine pyrophosphate, and sodium β -glycerophosphate were obtained from the Nutritional Biochemicals Corporation. Pyridoxamine phosphate and pyridoxal phosphate (97 to 103 per cent purity) were purchased from the California Foundation for Biochemical Research. Adenosine-5-phosphoric acid was obtained from the Schwarz Laboratories, adenosine triphosphate (potassium) from the Sigma Chemical Company, Inc., and pyridoxine hydrochloride from Merck and Company, Inc.

Samples of *p*-chloromercuribenzoic acid, γ -(*p*-arsenosphenyl)butyric acid, and *o*-iodosobenzoic acid were kindly supplied by Dr. L. Hellerman.

Methods—Male albino rats, 3 to 5 months old, were decapitated, and the kidneys were removed and homogenized in 40 ml. of distilled water with a Potter-Elvehjem tissue grinder. After 10 minutes of agitation with N₂ gas, 0.3 ml. of the homogenate (20 to 25 mg. of dry weight) was added to 20 ml. beakers containing 10 μ moles of 5-hydroxy-DL-tryptophan¹ in 1.0

[†] A brief report of this study was given before the American Society of Biological Chemists, April 15-19, 1957, in Chicago, Illinois.

¹ The following abbreviations are used throughout this paper: 5-hydroxy-DL-tryptophan, 5-HT; 5-hydroxytryptamine, 5-HTA; adenosine-5-phosphoric acid, AMP; adenosine triphosphate, ATP; 2,3-dimercaptopropanol (British anti-Lewisite), BAL.

ml of 0.1 N sodium pyrophosphate buffer, pH 8.0, previously gassed with nitrogen for 5 minutes, and the contents were made to a final volume of 3.0 ml with buffer. All the preparations were made at 0–3°. The reaction was carried out in a nitrogen atmosphere at 38° in a Dubnoff metabolic incubator.

At the end of the incubation, 3.0 ml of 10 per cent trichloroacetic acid were added, the supernatant fluid, after centrifugation, was appropriately diluted with borate buffer (9), and 5-HTA was determined spectrophotometrically or colorimetrically (9).

For the inhibitor studies, the homogenate described above was used. To the main compartment of a standard Warburg flask with a double side arm were added 0.3 ml of homogenate and 2.1 ml of pyrophosphate buffer containing sufficient inhibitor to give the indicated final concentration. After a 5 minute treatment with N₂ gas in the cold and a 10 minute temperature equilibration, 10 μ moles of 5-HT in 0.3 ml of buffer were tipped in from one side arm. The flasks were incubated at 38° for the indicated period after which the compound utilized to test reversal of inhibition (pyridoxal phosphate, cysteine, etc.) was added from the second side arm. Incubation was continued as stated. The reaction was stopped and analyses were carried out as described above.

Appropriate blanks were included when required. All data for 5-HTA formation are expressed in terms of dry weight of homogenates used.

RESULTS AND DISCUSSION

Under the conditions described, the optimal pH was 8.0 and the rate of formation of 5-HTA was a linear function of time (Table I) in agreement with data reported for 5-HT decarboxylase in guinea pig kidney (1). The addition *in vitro* of 500 γ (2.02 μ moles) of either pyridoxal phosphate or pyridoxamine phosphate resulted in greater than a 2-fold increase in activity. Pyridoxal hydrochloride and pyridoxine hydrochloride were ineffective in this system (Table II).

These results could have been related to destruction of a required endogenous organic phosphate during the experiment. Investigation demonstrated that neither phosphatase inhibitors (0.01 M fluoride or citrate present during homogenization and incubation) nor organic phosphates (30 μ moles of sodium β -glycerophosphate, 1.05 μ moles of thiamine pyrophosphate, 2.02 μ moles of AMP or 10 μ moles of ATP) significantly altered the 5-HT decarboxylase activity of rat kidney homogenate. Further study revealed that as little as 0.04 μ mole of pyridoxal phosphate (1.35×10^{-5} M) caused a maximal increase in activity with half maximal activation obtained at 2×10^{-6} M pyridoxal phosphate. Since the addition of 0.04 μ mole of pyridoxal phosphate caused an actual increase of 2 μ moles in 5-

HTA formation, the effect is catalytic, as expected of a cofactor. These findings indicate also that the observed stimulation of activity with either pyridoxal phosphate or pyridoxamine phosphate was not the result of non-specific protection of other organic phosphates which might be present in the homogenate. The possibility that endogenous phosphorylated pyri-

TABLE I
5-HTA Formation As Function of Time

Incubation time <i>hrs</i>	5 HTA formed, μ moles per 100 mg	
	Control	Pyridoxal phosphate added
2	2.38	6.02
4	5.02	11.92

The conditions were described under "Experimental." 2.02 μ moles of pyridoxal phosphate added when present.

TABLE II
Effect of Pyridoxine Derivatives on 5-HT Decarboxylase

Addition	No. of experiments	5 HTA formed, μ moles per 100 mg per hr	
		Mean \pm	Standard error
None	11	1.14 \pm	0.02
Pyridoxine hydrochloride	2	1.36	
Pyridoxal	2	1.36	
" phosphate	6	2.84 \pm	0.01
Pyridoxamine phosphate	2	2.82	

The conditions were as described under "Experimental." 500 γ (2.02 to 2.45 μ moles) of pyridoxine derivatives were added when present.

doxine derivatives were destroyed by other systems, *e.g.* by conversion to pyridoxic acid (10), during the experiment is not eliminated by these data.

The effect of supplemental pyridoxine in the diet was investigated. A group of rats was placed on the stock diet supplemented additionally with 20 mg per kilo of pyridoxine hydrochloride. After 7 to 10 days on this diet, the 5-HT decarboxylase activity of the kidneys of these rats was determined with and without the addition *in vitro* of pyridoxal phosphate. The results of typical experiments are presented in Table III. The 5-HT decarboxylase activity of the stock kidney was significantly increased by the administration of supplemental pyridoxine in the diet. This finding

is not unexpected in view of the report that the codecarboxylase content of rat tissue can be increased by feeding pyridoxine hydrochloride in excess of the amount required for optimal growth (5). The addition *in vitro* of pyridoxal phosphate caused an increase in the kidney 5-HT decarboxylase activity in both stock- and pyridoxine-supplemented rats, although, when compared to the corresponding control values, this increase was not as marked in the supplemented rats as in the stock rats. An increased endogenous supply of codecarboxylase in the supplemented rats (5) could have accounted for these results.

Hydroxylamine Inhibition and Reversal—Clark *et al* (1) have reported that, at pH 6.0, pyridoxal phosphate would not overcome semicarbazide inhibition of hog kidney 5-HT decarboxylase. In their study 0.81 μ mole of pyridoxal phosphate (3×10^{-4} M, assuming a final volume of 2.7

TABLE III
Effect of Supplemental Dietary Pyridoxine on 5-HT Decarboxylase

Diet	Pyridoxal phosphate added <i>in vitro</i>	5 HTA formed, μ moles per 100 mg per hr	Per cent increase
Stock	—	1.20	
"	+	3.02	152
" + pyridoxine	—	2.21	
" "	+	3.29	49

The conditions were as described under "Experimental." When present, 2.02 μ moles of pyridoxal phosphate were added.

ml) and 10^{-2} M semicarbazide were used. Although the pyridoxal phosphate was not added in stoichiometric excess, reversal of semicarbazide inhibition of dopa decarboxylase was obtained under these conditions (1). It was thought desirable to ascertain whether a true stoichiometric excess of the cofactor could overcome the inhibition of 5-HT decarboxylase by a carbonyl reagent. The results obtained with hydroxylamine (5×10^{-4} M) and pyridoxal phosphate (1×10^{-3} M) are presented in Table IV.

The addition of excess pyridoxal phosphate overcame the hydroxylamine inhibition of 5-HT decarboxylase. That these results are due, at least in part, to a true reversal and not merely to pyridoxal phosphate activation of the uninhibited portion of the enzyme can be seen from a consideration of the kinetics of the reaction. In the presence of 5×10^{-4} M hydroxylamine, 33.0 per cent of the enzymatic activity was unaffected by the inhibitor. This would be equivalent to 9.6 mg of dry weight of homogenate. From Table I it can be calculated that maximal activation of this amount of enzyme by pyridoxal phosphate would result in an additional 0.173

μ mole of 5-HTA per hour If the results of pyridoxal phosphate addition to the inhibited reaction (Table IV) were merely the result of activation of

TABLE IV
Effect of Pyridoxal Phosphate on Hydroxylamine Inhibition of Rat Kidney 5-HT Decarboxylase

Additions	5 HTA formed, μ moles per 100 mg per hr	
	Total	Net
1 Substrate only	1 00	1 00
2 (1) + NH_2OH (5×10^{-4} M)	0 33	0 33
3 (2) + pyridoxal phosphate (10×10^{-4} M)	1 31	1 21
4 Pyridoxal phosphate, no substrate (10×10^{-4} M)	0 10	

Conditions 2.1 ml of 0.1 M Na pyrophosphate buffer, pH 8.0, and 0.3 ml of homogenate (29 mg of dry weight) in the main compartment After gassing and equilibration (see "Experimental"), 10 μ moles of 5-HT were tipped in from one side arm and incubated for 1 hour Pyridoxal phosphate (3.0 μ moles) was then added from the second side arm and incubated for 1 hour Final volume 3.0 ml, incubated for a total of 2 hours in N_2 , 38°

TABLE V
Effect of BAL and Cysteine on Inhibition of Rat Kidney 5-HT Decarboxylase

Inhibitor (10^{-3} M)	5 HTA formation, μ moles per 100 mg per hr					
	Control		Cysteine		BAL	
	5 HTA	Per cent inhibition	5 HTA	Per cent inhibition	5 HTA	Per cent inhibition
None	2 58		2 80		2 43	
<i>p</i> -Chloromercuribenzoate	0 78	69 8	1 31	53 2	1 47	39 6
<i>o</i> -Iodosobenzoate	1 36	47 4	2 12	24 3	1 70	30 5
<i>p</i> -Arsenosphenylbutyrate	0 66	74 4	0 86	69 4	0 79	67 5

Conditions The conditions were described under "Experimental" Homogenate was prepared in cysteine or BAL to give a final concentration in the flask of 5×10^{-3} M Each beaker contained 0.10 μ mole of pyridoxal phosphate Incubated for 2 hours, 0.1 M pyrophosphate buffer, pH 6.6, in N_2 , 38° Similar results obtained at pH 8.0

the uninhibited portion of the enzyme, the net 5-HTA formed would have been 0.50 μ mole rather than the observed 1.21 μ moles It is clear that both reversal and activation occurred The inability of Clark *et al* to overcome such inhibition of hog 5-HT decarboxylase by the addition of pyridoxal phosphate may have been due partially to pH effects These

workers reported data from studies carried out at pH 6.0, where the activity of the enzyme was about one-third that at the optimal pH 8.0 (1)

Effect of Sulfhydryl Inhibitors—5-HT decarboxylase was inhibited by 10^{-3} M *p*-chloromercuribenzoate, *o*-iodosobenzoate, and γ -(*p*-arsenosphenyl)butyrate. These inhibitions could not be overcome or prevented by 5×10^{-3} M cysteine or BAL (Table V). Increased concentrations of substrate or of pyridoxal phosphate also failed to alter the inhibition due to *p*-chloromercuribenzoate. Since inhibition was not obtained with 10^{-3} M benzoate or cinnamate, these results are taken to indicate that the observed inhibitions were due to non-specific heavy metal or oxidative action rather than to specific involvement of the thiol groups.

Effect of Cations—Marked inhibition of 5-HT decarboxylase activity was obtained by 10^{-3} M cupric ions at both pH 6.6 and 8.0. Fe^{++} , Fe^{+++} , Mg^{++} , and Mn^{++} had no effect on the activity and, as already mentioned, neither 0.01 M fluoride nor citrate inhibited the reaction. These data cannot be interpreted as indicating that none of the cations tested are essential for the formation of 5-HTA since the ions could be present in non-limiting concentrations in the homogenates used.

SUMMARY

It has been demonstrated that rat kidney 5-hydroxytryptophan decarboxylase requires pyridoxal phosphate as a cofactor. Data are also presented which indicate that this enzyme does not require thiol groups for activity. Cupric ions were found to be inhibitory but several other cations were without effect on the reaction under the conditions used.

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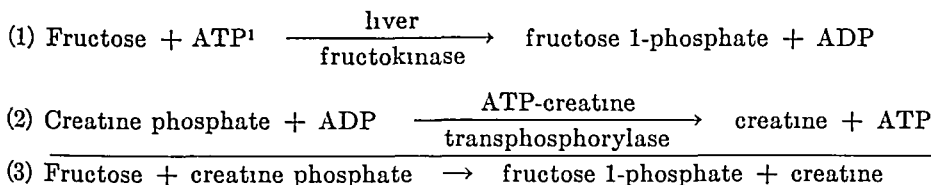
LIVER FRUCTOKINASE*

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An investigation of the fructokinase of liver was undertaken because of the importance of this enzyme for fructose metabolism and because of its interesting kinetic behavior. Heis (1) reported that the enzyme requires an unusually high concentration (1 to 2 M) of potassium ion for optimal activity. The enzyme was also found to respond anomalously toward oxygen. Vestling *et al.* (2) and Coll *et al.* (3) demonstrated that fructose phosphorylation is much greater in air than under anaerobic conditions. It was not immediately apparent how oxygen was involved in this process since the primary reaction is simply that shown in Reaction 1.



The possibility that ATP becomes limiting under anaerobic conditions was eliminated as the explanation of the decreased fructose consumption (3, 4). Previous workers seem not to have explored the possibility of product inhibition. One of the reaction products, fructose 1-phosphate, could be ruled out as the inhibitor because the addition of fluoride, which causes greater accumulation of fructose 1-phosphate, did not inhibit fructose utilization (3). To test the possibility that ADP inhibits fructokinase we added creatine phosphate and purified muscle ATP-creatine transphosphorylase (5) in non-limiting amounts to the liver fructokinase assay system. Under these conditions ADP is converted to ATP (by Reaction 2) as rapidly as it is formed. Preventing the accumulation of ADP in

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¹ The following abbreviations are employed: adenosine triphosphate, ATP, adenosine diphosphate, ADP, tris(hydroxymethyl)aminomethane, Tris.

this manner was found to enhance fructokinase activity greatly. Moreover, it brought about a proportionality between amount of enzyme and fructose disappearance, thus providing an accurate and reproducible assay for aid in purification. Since oxidative phosphorylation will also result in removal of ADP, it is likely that the effect of aerobic conditions on fructokinase activity results from the removal of this inhibitor.

With this assay it has been possible to purify beef liver fructokinase well beyond the 10- to 20-fold increase in specific activity reported by earlier workers (1, 6, 7). To date we have developed a reliable procedure for purifying the enzyme 150-fold. Some preparations have been carried through additional purification to 600-fold, but, even at this stage, 100,000 gm of the protein phosphorylate only 150 moles of substrate per minute at 30°. This report deals mainly with cofactor and kinetic studies.

Results

In confirmation of earlier work (2), we found in preliminary experiments with homogenates of rat liver that additions of an oxidizable substrate such as α -ketoglutaric acid doubled the rate of fructose disappearance at low enzyme concentration. The stimulation was even greater at higher enzyme concentrations, for in the presence of this substrate there was a nearly linear relationship between amount of homogenate added and fructose disappearance.

Additions of creatine phosphate and ATP-creatine transphosphorylase enhanced fructose disappearance even more than did the oxidizable substrate. No stimulation of fructokinase activity occurred when either creatine phosphate or the transphosphorylase was added separately.

Fig. 1 shows the effect of adding the creatine phosphate system at zero time and after 15 minutes. Rat liver homogenate (1.5 in 0.15 M KCl) was employed as the fructokinase source in this experiment. The response to added creatine phosphate was similar regardless of the time of addition. This is considered to be strong evidence that ADP is the only significant fructokinase inhibitor produced. In other experiments it was found that inclusion of the creatine phosphate system results in linearity between fructose disappearance and fructokinase increment in non-particulate liver preparations as it does with liver homogenates. This made purification of the enzyme feasible.

Cofactors and Kinetic Studies

All experiments which follow were performed with a fructokinase preparation purified approximately 400-fold from beef liver. 1 mg of protein in this preparation catalyzed the disappearance of about 1 μ mole of fructose per minute at 30° under optimal conditions.

Table I demonstrates the effect of various concentrations of twice recrystallized ATP-creatine transphosphorylase on fructokinase activity in the presence of 5 mM creatine phosphate. Maximal activation is produced

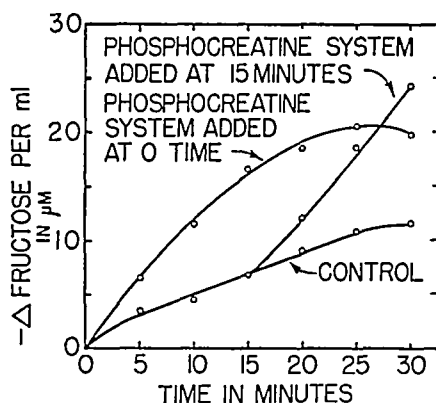


FIG 1 Effect of adding phosphocreatine system at different times. The reaction mixture contained 3.3 mM fructose, 5 mM $MgSO_4$, 16 mM potassium phosphate buffer, pH 7.3, 40 mM KF, 3.3 mM ATP, and 6.2 mM KCl. The phosphocreatine system consisted of 4 μ moles of creatine phosphate and 0.2 mg of ATP-creatine transphosphorylase per ml of reaction mixture. Incubated at 30°.

TABLE I

Role of ATP-Creatine Transphosphorylase in Maintaining Fructokinase Activity

ATP-creatine transphosphorylase concentration	Fructose disappearing in 10 min
units per ml	μ moles
0	0.29
0.001	0.30
0.01	0.40
0.10	0.88
1.0	1.03
5.0	1.08
10.0	1.08
100.0	1.09
100.0 (no fructokinase)	0

The system contained 2 μ moles of fructose, 2 μ moles of $MgSO_4$, 10 μ moles of phosphate buffer (potassium salts), pH 7.3, 2 μ moles of ATP, 5 μ moles of creatine phosphate, and 0.1 unit of purified fructokinase in a final volume of 1 ml. Incubation time 10 minutes at 30°.

by about 1 unit (20 γ) of the transphosphorylase per ml. Omission of fructokinase from the system abolished fructose disappearance even in the presence of 100 units (2 mg) of ATP-creatine transphosphorylase. Unless stated otherwise, 10 units (200 γ) per ml of the transphosphorylase were used routinely in all subsequent experiments.

The effect of varying the concentration of creatine phosphate on the activation of liver fructokinase is shown in Fig 2. Maximal activation was achieved with 5 mM creatine phosphate. In this experiment, slight inhibition from maximal activity occurred when more than 5 mM creatine phosphate was employed (see the inset of Fig 2). We were unable to explain this inhibition until the importance of maintaining an Mg^{++} ATP ratio of at least 1.0 was realized (to be discussed below). In this experiment both the ATP and Mg^{++} concentrations were 2 mM. Apparently, binding of Mg^{++} by high concentrations of creatine phosphate effectively lowered the Mg^{++} ATP ratio below the critical value of 1.0. When this

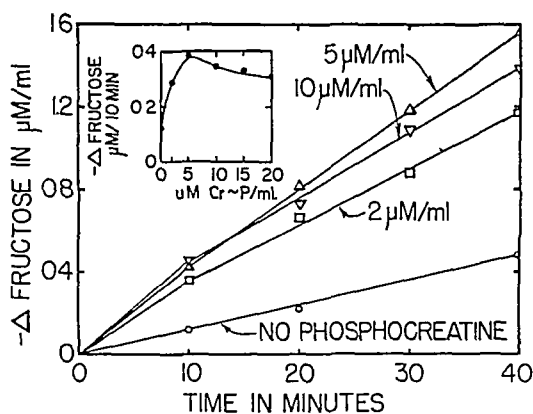


FIG 2

FIG 2 Stimulation of liver fructokinase by various concentrations of creatine phosphate. The reaction system was similar to that described in Table I except that 0.05 unit of fructokinase per ml was added.

FIG 3 Determination of apparent Michaelis constant for ATP. Phosphocreatine system present in non-limiting concentrations. 3 mM $MgSO_4$ to minimize effect of binding of Mg^{++} by creatine phosphate.

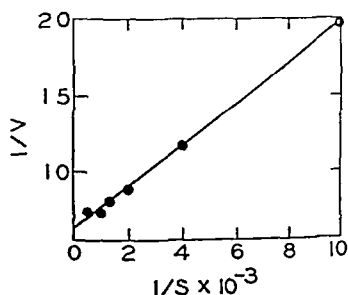


FIG 3

experiment was repeated with 5 mM Mg^{++} and 2 mM ATP, no inhibition of fructokinase occurred when creatine phosphate was added in excess of 5 μ moles per ml (8).

It was observed that the rate of fructose disappearance was approximately linear with time even when only 0.4 μ mole of fructose per ml remained in the reaction mixture. This confirms earlier observations (1) that the enzyme has a great affinity for fructose, i.e., the Michaelis constant for this sugar is less than 4×10^{-4} M.

The broad (pH 5.5 to 8) pH optimum reported by Hers (1) has been confirmed with the purified enzyme.

Nucleotide Requirement—The Michaelis constant (K_s) for the Mg^{++} -ATP complex was determined by Hers (9) employing a 20-fold purified preparation of beef liver fructokinase. He reported values of 5.4×10^{-3}

M in the absence of K^+ and 1×10^{-3} M in the presence of 1.0 M K^+ . These determinations were made in the absence of a mechanism for removing ADP.

When the K_s for ATP was determined with purified beef liver fructokinase, and with the addition of non-limiting amounts of Mg^{++} and the creatine phosphate system, a value of 2.1×10^{-4} M was obtained (Fig. 3). This is about 5 times lower than the lesser value reported by Heis (9). The creatine phosphate system was demonstrated to be non-limiting, since doubling its concentration had no effect on the reaction rate at low ATP concentrations.

When K_s is determined in this fashion, ATP is regenerated as it is con-

TABLE II
Inhibition of Fructokinase by ADP

Added ADP	Fructose disappearance in 20 min	
	5 mM ATP	10 mM ATP
M	μmole	μmole
0	0.58	0.56
0.005	0.18	0.20
0.010	0.11	0.11
0.015	0.05	0.08
0.020	0.00	0.02

The reaction mixture contained 20 mM K^+ , 50 mM Mg^{++} , 10 mM phosphate buffer, pH 7.4, 2 mM fructose, and 0.28 unit of beef liver fructokinase per ml. Creatine phosphate system omitted.

sumed and its concentration does not decrease significantly during the course of the reaction. Consequently the reaction rate does not decline progressively as it would with limiting amounts of ATP in the absence of an ATP-regenerating system. This makes possible the determination of very low K_s values for ATP or ATP-metal complexes, some of which were not measurable previously with the available assays.

Inosine triphosphate and uridine triphosphate in concentrations of 5 mM cannot replace ATP in the fructokinase reaction. A sample of crystalline ATP, in which no other nucleotides were demonstrated by paper chromatography, was fully active.

Inhibition by ADP—When ADP was added to the reaction mixture and the creatine phosphate system omitted, fructokinase activity was strongly inhibited (Table II). Increasing the ATP concentration from 5 to 10 mM did not reverse this inhibition. Enzymatic activity was completely suppressed by the presence of 20 mM ADP. It should be pointed out that the

inhibition occurring in this experiment was superimposed upon the already profound inhibition caused by omission of the creatine phosphate system

Monovalent Cation Requirement—Hers (9) was unable to demonstrate an absolute requirement for potassium or other alkali metal ions with a 20-fold purified preparation of beef liver fructokinase. However, a marked stimulation of enzymatic activity was encountered when the potassium concentration was increased to exceedingly high levels, *ie* 1 to 2 M depending upon the Mg^{++} and ATP concentrations. The more purified preparation described here has yielded markedly different results.

When all acidic reaction components were neutralized with Tris, the enzyme was without activity in the absence of potassium, certain alkali

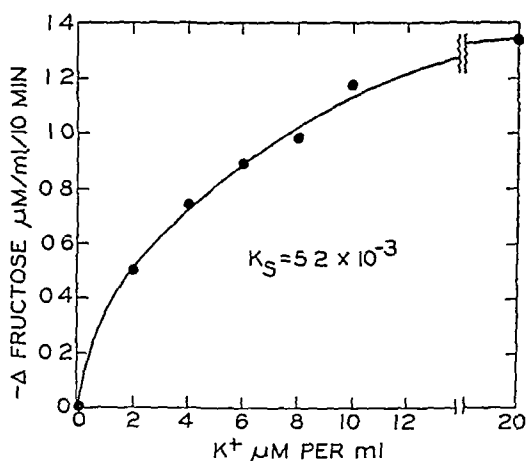


FIG 4 Requirement of liver fructokinase for K^+ . Tris salts of ATP and creatine phosphate were used. The reaction mixture contained 5 mM phosphocreatine, 5 mM ATP, 10 mM $MgSO_4$, 10 mM Tris sulfate, pH 7.3, and 2 mM fructose.

metal ions, or ammonium ions. Maximal activation was effected by 20 mM potassium (Fig. 4) and the Michaelis constant for this cation was 5.2×10^{-3} M. With the more purified enzyme the activation by potassium was found not to be affected by the Mg^{++} -ATP concentration as it was in Hers' work (9). Repetition of the experiment summarized in Fig. 4 but with 2 mM ATP and 4 μmoles of Mg^{++} gave identical results.

Monovalent cations other than potassium will also activate liver fructokinase with varying degrees of effectiveness (Table III). Rubidium is at least as effective as potassium, whereas sodium and ammonium ions activate the enzyme only about 70 per cent even in 0.5 M concentrations. Lithium and cesium ions activate only slightly in relatively high concentrations. We have not ruled out the possibility that the lithium and cesium samples used may be contaminated with trace amounts of potassium.

Magnesium-ATP Interrelationship—The observation first reported by

great as 5-fold the concentration of ATP, did not inhibit. However, excess ATP is strongly inhibitory (Fig. 5). In fact, complete inhibition of fructose disappearance occurred with an Mg^{++} :ATP ratio of 1:5. This is further evidence in support of Hers' (9) suggestion that the actual phosphate donor is a magnesium-ATP chelate, although present evidence does not indicate which of the possible chelate forms is the active substrate. There are several possible mechanisms by which excess ATP might inhibit the phosphorylation of fructose. Free ATP might act directly as a competitive inhibitor of the ATP-magnesium chelate which is the active substrate. Excess ATP might bind the Mg^{++} in an inactive, or inhibitory, chelate form containing a higher ratio of ATP to Mg^{++} than is found in the active chelate. A third possibility is that the enzyme is activated by free Mg^{++} and that addition of excess ATP results in removal of the Mg^{++} by chelation.

DISCUSSION

The marked discrepancy in the response of liver fructokinase to K^+ reported by Hers (9) with that observed in the present study has not been explained. It seems probable that the difference in the purity of the enzyme preparations used is somehow accountable. A complicating factor may have been eliminated in the course of purifying the protein to a specific activity greater than that used by Hers.

The study of inhibition of liver fructokinase by ADP is a complicated one. First of all, the experiments must be performed in the absence of an ATP-regenerating system. Therefore, the fructokinase is operating at a fraction of maximal efficiency, even when no inhibitor is added. Also, analysis of the system is made difficult by the fact that the concentration of the inhibitor, ADP, increases as the reaction proceeds. ADP, when added to the reaction mixture, seems to be a powerful non-competitive inhibitor (Table II). However, with the purified enzyme, the initial reaction rate is maintained (Fig. 2) even when the creatine phosphate system is omitted. Under these conditions the reaction rate is markedly inhibited from the onset when only minute amounts of ADP are present, *i.e.*, there is no need to accumulate ADP to produce significant inhibition. This suggests that ADP generated at the enzyme surface is a more powerful inhibitor than that which is added to or accumulates in the reaction mixture.

The striking inhibitions of fructokinase activity by ADP, and by ATP in excess of equimolarity with the Mg^{++} present, may play important roles in the regulation of metabolic processes. Inhibition of enzymatic reactions by a product is a common phenomenon. The ADP-fructokinase relationship parallels the inhibition of hexokinase by glucose 6-phosphate described by Crane and Sols (10). The inhibition of an enzymatic reaction

by an excess of substrate is not an unusual phenomenon (11) The unique aspect of the inhibition by ATP is the fact that, in the Michaelis region, slight alterations of ATP or Mg^{++} concentration may have relatively great effects on reaction velocity This behavior suggests the possibility that control of cellular enzymes may be exerted under normal conditions by the alterations of ATP or Mg^{++} concentrations associated with various physiological processes Several ATP-requiring enzymes are susceptible to this type of inhibition (12) and it seems likely that more will be found to be similarly affected

EXPERIMENTAL

Materials and Methods

The nucleotides were the best grade available from the Pabst Laboratories or Sigma Chemical Company Crystalline sodium phosphocreatine was synthesized by a modification (13) of the original method of Enno and Stocken (14) For studies of the role of monovalent cations, the barium salts of creatine phosphate and of the nucleotides were converted to the Tris salt by the addition of Tris sulfate The solutions were adjusted to pH 7.4 by addition of Tris in the free base form Solutions of the sodium salts of nucleotides were brought to the same pH with 2 N NaOH All other reagents were c.p. or analytical grade

Twice recrystallized ATP-creatine transphosphorylase was prepared from rabbit muscle by Procedure B of Kuby, Noda, and Lardy (5)

Enzymatic activity was determined by measuring the disappearance of free fructose (15) from the reaction mixture after deproteinization with barium hydroxide-zinc sulfate (16) For assaying activity during the isolation procedure, optimal conditions for fructokinase and an excess of the ATP-creatine transphosphorylase were employed The reaction mixture contained sodium ATP, 0.004 M, Mg^{++} , 0.02 M, K^+ , 0.02 M, NaF, 0.062 M, glycylglycine buffer, pH 7.3, 0.12 M, creatine phosphate 0.01 M, and 10 units of ATP-creatine transphosphorylase per ml A unit of fructokinase is defined as that amount of enzyme which catalyzes the phosphorylation of 1 μ mole of fructose in 1 minute at 30° under conditions of maximal activity Protein was determined by the biuret method in all steps of purification except that the nitrogen content of the liver homogenate was determined by nesslerization after perchloric acid digestion Specific activity is defined as units of fructokinase per mg. of protein

Purification of Fructokinase

Extraction—The initial studies were performed with rat liver homogenate prepared in 0.15 M KCl These preparations catalyzed the disappearance of about 2.5 to 5.0 μ moles of fructose per minute per gm. of liver

Beef liver was used as the source of fructokinase for purification because it is available in large quantity. It has about one-fifth as much fructokinase activity per gm. as does rat liver. The liver is sliced and chilled in ice as soon as it is removed from the slaughtered animal. The slices are freed of fat and connective tissue and repeatedly washed in ice water to remove blood. 1 kilo of liver is passed through a meat chopper and stirred with 3 liters of 0.15 M phosphate buffer, pH 7.8, at $+2^{\circ}$ for 2 hours. The extract, collected by filtration through cheesecloth, is brought to pH 5.5 with cold 5 N acetic acid (about 80 ml.) and the precipitated protein is removed by centrifugation. The protein solution is passed through cheesecloth to remove particles and is then brought to pH 6.0. Because the quantity of fructokinase in different beef livers varies greatly, from 400 to 750 units are recovered at this stage. The specific activity is about 0.014 which represents a 3.9- to 5.7-fold purification over the liver homogenate.

A wide variety of procedures for extracting the enzyme has been tried. That described above has given the greatest yields of enzyme and highest specific activities.

First Methanol Fractionation—The extract is mixed with one-ninth its volume of 0.5 M potassium phthalate, pH 6.0, and the mixture is brought to a final concentration of 40 per cent methanol by slow addition of 80 per cent methanol cooled to -10° . During the addition, the temperature of the protein solution is kept below $+5^{\circ}$. The mixture is then warmed (in a 40° bath) to 20° and held at 20° until the denatured protein settles and the solution clears. This usually requires 10 to 15 minutes. The precipitate, which contains little or no activity, is removed by centrifugation.

The methanolic solution is then placed in a bath at -15° and stirred. When the temperature reaches -10° , 1.5 volumes of 90 per cent methanol (at -10°) are added slowly to bring the final methanol concentration to 70 per cent. The mixture is held at -10° for an hour and the precipitate is collected by centrifugation or gravity filtration at -10° . The enzyme is extracted from the precipitated proteins by stirring repeatedly with 0.05 M phosphate, pH 7.0, in such portions that the methanol concentration of the extracting liquid will not drop below 15 per cent. The fourth extraction is made with 0.05 M phosphate containing 15 per cent methanol. The volume of the combined extracts should approximate one-fourth to one-fifth of the volume of the original liver extract. This fractionation accomplishes a 14-fold purification over the original extract with a recovery of 85 to 90 per cent of the activity. The specific activity is about 0.20.

Glycogen Digestion—The extract is brought to approximately pH 7 with 1 N KOH and 0.3 gm. of NaCl is added. 4 ml. of clarified saliva are added and the solution is warmed to 30° . After keeping the solution at this temperature for 15 minutes, it is chilled to 0° .

Second Methanol Fractionation—The solution is adjusted to pH 6.0 with 5 N acetic acid and one-ninth of its volume of 0.5 M potassium phthalate buffer, pH 6.0, is added. The methanol concentration in the solution is computed by taking into account the added KOH, saliva, acetic acid, and phthalate solutions. The methanol content is then brought to 40 per cent by the addition of 80 per cent methanol as is described for the first step above. The mixture is kept at 0° for 1 hour before centrifuging to remove the precipitate which is discarded. The supernatant solution is cooled to -10° and the precipitate which forms is collected by centrifuging. It contains a small part of the total activity with a specific activity of 0.05 to 0.10 and can be saved and added to subsequent batches. The methanol content of the solution is brought to 60 per cent by adding two-thirds of a volume of 90 per cent methanol at -10°. After standing overnight at -10°, the precipitate is collected and dissolved in sufficient 0.05 M phosphate buffer to make approximately 100 ml. This second methanol step results in a 1.8- to 2-fold purification (specific activity 0.30 to 0.40). About 54 per cent of the original activity is recovered. If a fraction is taken between 54 and 60 per cent methanol, about 30 per cent of the total yield can be obtained with a specific activity of 0.38 to 0.42. The procedure to this stage is readily reproducible and results in about 150-fold purification. Further purification to specific activity of 1.5 has been achieved by fractionation with ethanol in the presence of 0.1 M magnesium acetate and with ammonium sulfate. The latter procedures have not given consistently reproducible purification. They will therefore not be reported until they have been studied sufficiently to permit description of optimal conditions.

SUMMARY

- 1 A procedure is described for the purification of beef liver fructokinase.
- 2 The enzymatic activity is strongly and non-competitively inhibited by a product of the reaction, adenosine diphosphate (ADP). Addition of a phosphate-donating system which continually removes ADP increased fructokinase activity several fold and permitted reliable estimation of enzymatic activity.
- 3 The purified enzyme has an absolute requirement for both monovalent and divalent cations. When Mg^{++} fills the requirement for divalent cation, K^+ and Rb^+ are the most active monovalent cations, NH_4^+ , Na^+ , Cs^+ , and Li^+ are decreasingly active while $tris(hydroxymethyl)amino-methane$ is inactive. Maximal activity was obtained with 0.02 M K^+ .
- 4 For optimal activity the concentration of Mg^{++} must equal or exceed the concentration of adenosine triphosphate (ATP). Excess ATP is so strongly inhibitory that the reaction velocity is brought to zero when the

ratio ATP/Mg reaches 5:1. The possible role of this type of inhibition in the regulation of metabolic processes is discussed.

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THE CONCOMITANT RELEASE OF ADENOSINE TRIPHOSPHATE AND CATECHOL AMINES FROM THE ADRENAL MEDULLA

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In the specific granules of the adrenal medulla, large amounts of adenosine triphosphate (ATP) are present together with the catechol amines (adrenaline and noradrenaline) (1, 2). Stimulation of the adrenal medulla via its secretory nerves is followed by a decrease in both the ATP and the catechol amines (3).

The experiments reported below were carried out in an attempt to elucidate further what happens to the ATP after stimulation of the adrenal medulla.

EXPERIMENTAL

Male sheep (body weight, 20 to 25 kilos) were chosen as experimental animals. The adrenal medulla of sheep can be easily separated from adrenocortical tissue. The amount of adrenal medullary tissue obtained from one animal is enough for cell fractionation. Stimulation of the adrenal medulla was done by means of insulin (Vitrum, Ltd, 12 i u per kilo of body weight intramuscularly). As is well known, the stimulating effect of insulin on the adrenal medulla is mediated by the sympathetic nerves.

Three experiments were performed, and in each two animals were used. One animal was given insulin, and the other served as a control. The insulin was given after 36 hours fasting (water was given *ad libitum* throughout). Between 17 and 20 hours after the injection, the animals were killed by a blow on the head and subsequently bled. Hypoglycemic symptoms were noted about 12 hours after the insulin injection, although no convulsions occurred. Animal 6 (Table I) died about 17 hours after the injection of insulin. The adrenals were taken out and chilled with ice as soon as possible (12 to 15 minutes after death, except for Animal 6, in this case the interval in question is not exactly known but did not exceed 80 minutes). The adrenal medulla was separated from the cortical tissue, weighed, and homogenized in 5 ml of 0.3 M sucrose (4). The samples were kept cool in ice throughout the preparations.

Cell Fractionation—The homogenate was freed from cells, nuclei, con-

nective tissue, etc., by centrifugation at $800 \times g$ for 4 minutes. The sediment (Fraction S1) was extracted with 0.4 N chilled perchloric acid (PCA). The extract was analyzed for catechol amines and proteins as well as for inorganic and acid-labile phosphate. The supernatant fluid was spun at $7000 \times g$ for 60 minutes at $+1^\circ$. The sediment (Fraction G1) containing the specific medullary granules and the supernatant fluid (Fraction A1) containing the extragranular cytoplasm, including the "microsomes," were also treated with chilled PCA (final concentration 0.4 N).

Assay Methods—Inorganic (P_0) and acid-labile phosphate (P_s , hydrolysis at 100° in N HCl for 8 minutes) was determined according to Fiske and Subbarow (5). The total amount of adrenaline and noradrenaline was assayed according to the colorimetric method of von Euler and Hamberg (6). The PCA precipitates were analyzed for protein according to the biuret method of Cleland and Slater (7).

Ion Exchange Chromatography—The pH of the PCA extract of Fraction G1 was immediately adjusted to about 7 with chilled KOH. The precipitate ($KClO_4$) was washed with distilled water, which was then added to the extract. An aliquot of the neutralized extract was passed through a cation exchange column (Dowex 50, 150 to 300 mesh, 30 mm \times 80 sq mm) in order to remove the catechol amines and other interfering material. In Experiment 1 (Animals 1 and 2) the hydrogen form of the ion exchange resin was used. It was found, however, that adenosine and adenosine monophosphate (AMP) were taken up by the hydrogen form. Later on, this was avoided by passing an acetate-acetic acid buffer (pH 6.5) through the column before use. The pH of the effluent was adjusted to 8.5 with ammonium hydroxide. The sample was then passed through an anion exchange column (Dowex 2, chloride form, 200 to 400 mesh, 20 mm \times 25 sq mm). The adenosine phosphates were eluted according to Cohn and Carter (8) at the rate of 0.2 to 0.3 ml per minute. 7×10 ml of 0.003 M HCl, 7×10 ml of 0.02 M NaCl in 0.01 M HCl, 5×10 ml of 0.2 M NaCl in 0.01 M HCl. The ultraviolet absorption of the various fractions was read in a Beckman spectrophotometer, model DU.

Results

Presentation of Data—As suggested by the data of Table I, it seems possible that the long lasting stimulation caused an increase in the weight of the adrenal medulla. The results therefore appear somewhat different when expressed in absolute terms or according to tissue weight. For this reason both types of presentation are given.

Catechol Amines—The insulin treatment caused a marked drop in the catechol amine content of the adrenal medulla (Table I). On the other hand, the effect of stimulation on the intracellular distribution of the cate-

chol amines was slight, if any. In the control animals the extragranular cytoplasm (Fraction A1, Table I) contained 14 to 17 per cent of the catechol amines of the total cytoplasm (Fraction (A1 + G1)), the corresponding figure for the stimulated animals (Nos 2 and 4) being 22 and 24 per cent (the high figure observed in Animal 6 may be due to postmortal changes (see below and Hillarp *et al* (4)). It would thus seem that no marked accumulation of catechol amines occurred in the extragranular cytoplasm during stimulation. Most of the extragranular catechol amines have probably been released from the granules after death (4). It does not seem unreasonable that the granules of heavily stimulated cells have an increased fragility and are thus more easily broken during homogenization. This would explain the somewhat higher values observed in the stimulated animals.

ATP and Its Split Products—In the medullary granules (Fraction G1) the acid-labile P dropped to practically the same degree as the catechol amines. (For obvious reasons this will be true, whether or not stimulation caused a real increase in the weight of the adrenal medulla.) As usual, no acid-labile P could be detected in the extragranular cytoplasm. However, this is of little significance, since any ATP or adenosine diphosphate (ADP) present outside the granules will probably be decomposed enzymatically during the cell fractionation, if not earlier (9).

In Animals 1 to 4 the adenosine phosphates of the medullary granules were fractionated by ion exchange chromatography. Most of the material with an adenine spectrum appeared in a fraction eluted promptly by 0.2 M NaCl in 0.01 M HCl (Fig. 1). This is in full agreement with the earlier experiments on cows, in which this fraction was identified as ATP (10). There was also good agreement between the ultraviolet absorption data and the values for the acid-labile phosphate.

Stimulation by insulin was followed by a marked decrease in the ATP fraction. This decrease was practically equal to the drop in acid-labile P. It was not accompanied by the appearance of a corresponding amount of ADP or AMP. Neither could any adenosine, which in Experiment 2 would have been present in the effluent from the anion exchange column, be detected. The loss of the adenine moiety of the ATP molecule from the granules was confirmed in Experiment 2 by reading the ultraviolet absorption spectra of the neutralized PCA extracts and subtracting the extinctions accounted for by the catechol amines (calculated from analyses of the catechol amine contents and the ultraviolet absorption spectra of known pure catechol amine solutions). In this way absorption spectra with peaks at 257 to 260 m μ were obtained (data not shown). The values for total adenine calculated from these curves were in good agreement with the data on ion chromatography.

No significant accumulation of adenine compounds could be detected in

TABLE 1
Content and Intracellular Distribution of Catechol Amines, Acid-Labile and Inorganic P, and Proteins in Adrenal Medullae of Normal and Insulin-Treated Sheep

Fraction S1, sediment after centrifugation at $800 \times g$ for 4 minutes (connective tissue, whole cells, cell nuclei, etc.), Fraction G1, sediment after centrifugation at $7000 \times g$ for 60 minutes (adrenal medullary granules), Fraction A1, supernatant fluid after last centrifugation (extragranular cytoplasm, including "microsomes")

	Experiment 1				Experiment 2				Experiment 3			
	Animal 1 (control), weight 25 kilos, adrenal medulla 242 mg		Animal 2 (insulin treated), weight 25 kilos, adrenal medulla 401 mg		Animal 3 (control), weight 22 kilos, adrenal medulla 198 mg		Animal 4 (insulin treated), weight 22 kilos, adrenal medulla 223 mg		Animal 5 (control), weight 25 kilos, adrenal medulla 268 mg		Animal 6 (insulin treated), weight 20 kilos, adrenal medulla 343 mg	
	γ	per cent total	γ	per cent total	γ	per cent total	γ	per cent total	γ	per cent total	γ	per cent total
Catechol amines												
Fraction S1	355	13	185	14	305	14	127	15	605	18	170	20
" G1	1950	73	905	68	1540	71	535	64	2280	70	395	45
" A1	365	14	250	19	315	15	168	20	385	12	310	35
Total	2670		1340		2160		830		3270		875	
Total, mg per gm medulla												
Fraction A1, % Fraction (A1 + G1)	11 0		3 35		10 9		3 72		12 2		2 55	
Degree of homogenization, %	16		22		17		24		14		44	
	87		86		86		85		82		80	
Acid-labile P												
Fraction S1, γ	29 0		17 6		24 5		12 0					
" G1, γ	166		87 5		120		49 0					
" " , γ per gm medulla†	790		254		710		258					
Inorganic P												
Fraction S1	13 3	9	20 3	10	10 1	8	10 7	10				
" G1	23 8	16	29 6	14	19 5	16	15 6	14				
" A1	114	75	162	76	94	76	85	77				
Total	151		212		124		111					

Total, γ per gm. medulla	625		530		630		500			
	mg		mg		mg		mg		mg	
Protein N	1 32	39	2 11	38	1 15	40	1 30	40	1 42	38
Fraction S1	0 94	28	1 24	22	0 82	28	0 70	22	0 95	26
" G1	1 14	34	2 25	40	0 91	32	1 22	38	1 35	36
" A1	3 40		5 60		2 88		3 22		3 72	
Total	14 1		14 0		14 5		14 4		13 9	
Total, mg per gm medulla	45		35		47		36		41	
Fraction G1, % Fraction (A1 + G1)	55		65		53		64		59	
Fraction A1, % Fraction (A1 + G1)										
Difference, insulin treated less control										
Catechol amines	Per cent per unit weight adrenal medulla	Per cent per 2 adrenal medullae	Per cent per unit weight adrenal medulla	Per cent per 2 adrenal medullae	Per cent per unit weight adrenal medulla	Per cent per 2 adrenal medullae	Per cent per unit weight adrenal medulla	Per cent per 2 adrenal medullae	Per cent per unit weight adrenal medulla	Per cent per 2 adrenal medullae
Fraction G1†	-72	-54	-69	-65	-86	-83	-73	-86	-83	-73
Total	-70	-50	-66	-62	-79	-73	-79	-79	-73	-73
Acid labile P	-68	-47	-64	-59	-32	-14	-14	-32	-14	-14
Fraction G1†	-20	+32	-23	-14	-32	-14	-14	-32	-14	-14
Protein N	+19	+97	+19	+35	-15	+9	+35	-15	+9	+9
Fraction G1†	±0	+65	±0	+12	-14	+11	+12	-14	+11	+11
" A1†										
Total										

* 100 less percentage of catechol amines in Fraction S1

† Corrected for incomplete homogenization

the cytoplasm outside the granules of the stimulated medullae. Apparently the adenine part of the lost ATP had disappeared from the adrenal medullary cells.

The possible splitting of ATP at stimulation might result in an accumulation of inorganic phosphate. However, no consistent change in the inorganic phosphate is apparent from the present data, either in the adrenal

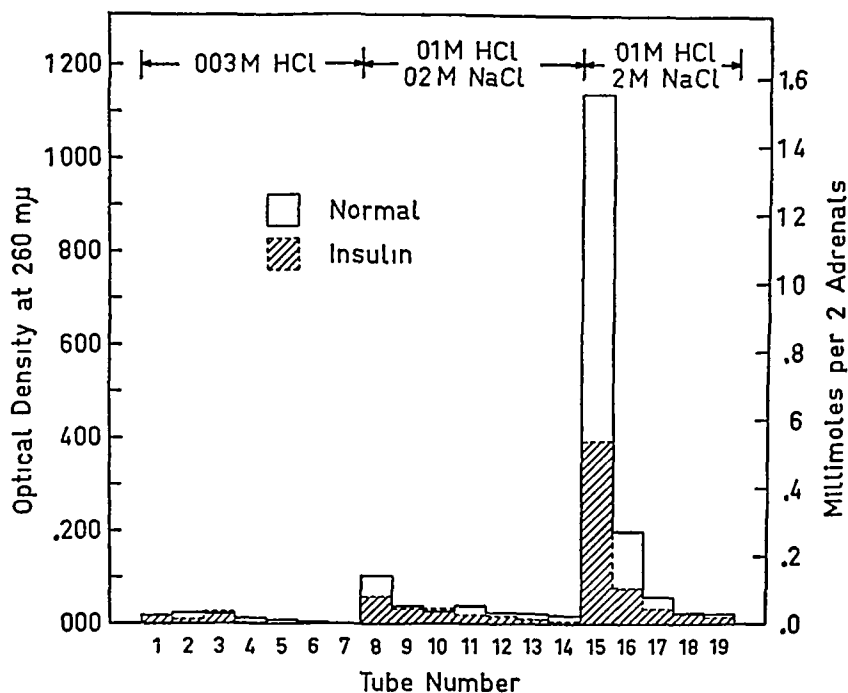


FIG 1 Ion exchange chromatography of adenosine phosphates of adrenal medullary granules of a normal and insulin-treated sheep. The chromatography was performed according to Cohn and Carter (8) after removal of the catechol amines by cation exchange. Equal aliquots of the two extracts were used for chromatography. The optical density at 260 $m\mu$ of the ATP fraction was 65 per cent lower in the insulin treated animal. Practically the same difference in acid-labile P and catechol amines was observed (Table I, Animals 3 and 4).

medulla as a whole or with respect to the intracellular distribution. The values observed are in essential agreement with those of normal cows (1).

Proteins—Stimulation did not apparently affect the protein content per unit weight of adrenal medulla, except for a possible slight decrease in Animal 6. Thus, if stimulation caused a real increase in the weight of the adrenal medulla (see above), there was not merely an accumulation of water but a parallel increase in proteins. There seemed to be a definite effect on the intracellular distribution of proteins. In the control animals the granule proteins constituted 41 to 47 per cent of the total cytoplasm proteins (Fraction (A1 + G1)), which is in agreement with earlier findings

in cows (11), whereas the corresponding range after insulin treatment was 35 to 36 per cent. This difference may be interpreted in different ways. If stimulation caused an increased formation of proteins, the newly formed proteins may have been present outside rather than inside the granules. Another possibility would be that part of the soluble granule protein was released into the extragranular cytoplasm. Experiments of shorter duration will probably be necessary to eliminate the possible influence of new protein formation. In fact, such an experiment has already been carried out in rats (3). In this experiment a similar but much less marked change in the intracellular distribution of proteins was observed. Such data may help to answer the question whether a damage of the granule membrane is responsible for the release of catechol amines after stimulation. The results of the experiment with rats argue definitely against a profound

TABLE II

Loss of Granule Protein (per Gm. of Medulla) after Stimulation

In each experiment, the observed difference between the control and the insulin-treated animal is given. This value is compared to the calculated loss, on the assumption that the loss of catechol amines is due to a destruction of the granule membrane.

Experiment No	Observed loss	Calculated loss
	<i>per cent</i>	<i>per cent</i>
1	20	36
2	23	34
3	32	43

damage of the granule membrane, since only a small and perhaps insignificant amount of proteins was lost from the granules of the stimulated adrenal medullary cells. The present data are less conclusive but point in the same direction. This is shown in Table II, in which the observed drop in granule protein is compared with the expected decrease, calculated on the assumption that the release of catechol amines was due to a destruction of granule membrane and thus accompanied by a complete loss of soluble proteins (the soluble proteins were assumed to constitute 50 per cent of the total granule proteins, according to analyses on cow and sheep granules).

DISCUSSION

In the medullary granules the molar ratio of catechol amines to ATP is not much above 4:1, which corresponds to equivalent amounts of acid and base. Although the physiological significance of this finding is not yet understood, it suggests an important role of ATP in the storage and release of the catechol amines. The present observations give further

support to this view. In accordance with the earlier experiments on rats and cats (3), stimulation of the adrenal medullae of sheep caused a drop in the ATP of the granules which was proportional to the decrease in catechol amines.

The important question as to whether ATP is split before being released from the granules is still open. In the granules no accumulation of ADP, AMP, adenosine, or inorganic P was detectable, but this does not permit any definite conclusions, since the hypothetical split products may have been promptly released along with the catechol amines. The medullary granules contain a very active adenosinetriphosphatase, which, however, does not attack the ATP inside the intact granules, although ATP added to the suspension medium is rapidly hydrolyzed to AMP (unpublished experiments). It is tempting to speculate that this enzymatic mechanism is involved in some way or other in the release of catechol amines. Perhaps stimulation enables the adenosinetriphosphatase to act on the ATP inside the granules.

Stimulation of the adrenal medulla did not seem to be followed by any marked accumulation of catechol amines, adenine derivatives, or inorganic P in the cytoplasm outside the granules. This would indicate that both catechol amines and ATP (or its split products) leave the cells rather promptly after being released from the granules. However, such an accumulation might perhaps be detectable in experiments of shorter duration.

The problem of a change in the granule membrane at stimulation is still unsolved. Although the data so far available argue against a profound damage of the granule membrane, they seem to indicate a certain loss of protein from the granules. However, in view of the sources of error discussed above, it would be premature to do more than suggest such an effect.

Electron microscopic evidence that the granule membrane is still present after stimulation has been supplied by Wetzstein, who kindly showed us some of his not yet published electron micrographs. In another electron microscopic study Sjöstrand and Wetzstein (12) conclude that the adrenal medullary granules constitute about 3.5 per cent of the cytoplasm. This figure was found in experiments on mice. In guinea pigs and cats the granules were said to present a very similar picture. The corresponding figure observed by us in cows and sheep with use of fractional centrifugation is several times higher. This discrepancy is thought by Sjöstrand and Wetzstein to be due possibly to a large contamination of the granular fraction with "other cell constituents (for instance possibly also mitochondria)" in the centrifugation experiments. However, unless the adrenal medullae of mice are exceptionally poor in catechol amines, it can easily be calculated that their figure is unreasonably low. The catechol amine content of the whole adrenal medulla is 1.2 per cent (in cows, we have observed

similar values in sheep and rats) This means that the catechol amine content of the cytoplasm, *i.e.* the whole adrienal medulla *minus* nuclei, connective tissue, blood vessels, etc., can hardly be below 17 per cent Since practically all the catechol amines of the adrienal medulla are present in the granules, the catechol amine content of the granules would then be about 50 per cent ($(17 \times 100)/35$ per cent) The ATP content of the granules is about half the catechol amine content These two components together would thus constitute 75 per cent of the granules Sjostrand and Wetzstein state that the granules contain a protein framework and a membrane If this protein is also taken into account, the granules would consist almost entirely of solids and thus contain little or no water With such a low water content the sedimentation characteristics of the granules would be entirely different from those actually observed In fact, there is nothing to suggest an unusually low water content of the granules Unless marked species differences exist, it is hard to avoid the conclusion that a considerable shrinkage of the granules has occurred in the preparations of Sjostrand and Wetzstein This will also explain the wide empty spaces surrounding the granules in these preparations The explanation offered by Sjostrand and Wetzstein that these spaces have been occupied by lipides which have been dissolved during the preparation seems unlikely in view of the fact that the adrienal medulla is not unusually rich in lipides (11) The assumption of a shrinkage is further supported by electron microscopic measurements on direct smears, in which case the granules were found to be much larger than those in the fixed preparations of Sjostrand and Wetzstein (12) Finally, as to the possible contamination of the granular fraction with other cell constituents, no such components are visible in the pictures of Sjostrand and Wetzstein, except for a very small number of mitochondria This is in good agreement with our earlier work on the purity of the granular fraction (4)

Recently a short note by D'Iorio and Eade (13) appeared in which it was stated that insulin treatment failed to cause any decrease in the ATP content of the adrenals of rabbits As no details are given, no definite explanation of the discrepancy between their results and ours can be offered However, the analyses seem to have been made on whole adrenals, as suggested by the low ratios of adrenaline to protein nitrogen given in a table In view of the fact that in the adrenals of rabbits the amount of medulla is small compared with the amount of cortical tissue, the ATP of the cortical tissue may have masked changes in the medullary ATP

SUMMARY

The adrenal medullae of sheep were stimulated by insulin, the action of which is mediated by the sympathetic nervous system

Stimulation was followed by a decrease in the adenosine triphosphate

(ATP) stored in the adrenal medullary granules, which also contain the catechol amines (adrenaline and noradrenaline) The decrease in ATP was proportional to the simultaneous drop in catechol amines There was no corresponding accumulation of ATP in the extragranular cytoplasm or of split products such as adenosine diphosphate, adenosine monophosphate, adenosine, or inorganic phosphate either in the granules or in the extragranular cytoplasm

As indicated by data on the intracellular distribution of proteins, the concomitant release of ATP and catechol amines does not seem to be due to a destruction of the granule membrane

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ERYTHROCYTE METABOLISM

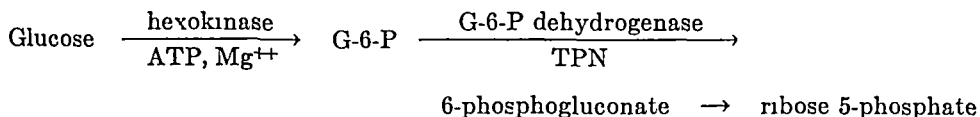
III OXIDATION OF GLUCOSE*

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Although mature (non-nucleated) human erythrocytes metabolize glucose principally to lactic acid via the glycolytic scheme (2-4), considerable evidence has been obtained by Warburg *et al* (5) and others that these cells also contain the enzymes of the "phosphogluconate oxidation" pathway (6-8). In the initial phase of this pathway, glucose is converted oxidatively to ribose 5-phosphate, according to the following diagram ¹



TPNH, produced by the G-6-P dehydrogenase reaction, is oxidized by molecular oxygen (9)

Harrop and Barron (10), Michaelis and Salomon (11), and Warburg *et al* (5) first called attention to the interesting fact that the extremely low level of glucose oxidation in mature erythrocytes is stimulated 20- to 50-fold in the presence of catalytic quantities of methylene blue and certain other dyes, thus suggesting that the oxidation of TPNH is limited ordinarily in the cell only by the deficiency of an electron carrier or coenzyme. Kiese (12) later demonstrated that glucose oxidation in erythrocytes can be linked also to the reduction of methemoglobin² and that this process, too,

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¹ The following abbreviations are used DPN and TPN, di- and triphosphopyridine nucleotides, DPNH and TPNH, reduced DPN and TPN, ATP, adenosine triphosphate, G-6-P, glucose 6-phosphate, MeB, methylene blue, ACD, acid-citrate-dextrose

² Carson *et al* (13) have shown that erythrocytes of primaquine-sensitive individuals are deficient in G-6-P dehydrogenase. The resultant decrease in the level of endogenous TPNH then prevents these cells from reducing methemoglobin (produced by chemical oxidation of hemoglobin by primaquine) at the normal rate.

is stimulated greatly by methylene blue. In hemolyzed preparations, however, these early investigators found that the oxidation of glucose by either oxygen or methemoglobin is absent, even in the presence of methylene blue (9-11).

The present investigation was undertaken in order to extend the above findings on the aerobic oxidation of glucose in erythrocytes and, in particular, to obtain information on the electron transport system linking TPNH to oxygen in the presence of methylene blue.

EXPERIMENTAL

Materials—DPN, TPN, DPNH, ATP, and G-6-P were obtained from the Sigma Chemical Company. Hexokinase was a product of the Pabst Laboratories, and catalase was obtained from the Worthington Biochemical Corporation. TPNH was prepared by reduction of TPN with hydro-sulfite or with the isocitric dehydrogenase system (14). G-6-P dehydrogenase was prepared from yeast by the method of LePage and Mueller (15).

Preparations—Human blood, collected in ACD (National Institutes of Health, Formula B), was made available through the cooperation of Dr. Richard Czajkowski of the King County Central Blood Bank. The blood was stored at 4° for not longer than 4 days before being used in experiments. Whole cell samples were prepared by removing the plasma by centrifugation for 30 minutes at $2300 \times g$ in the International refrigerated centrifuge, model PR-1, washing the cells twice by suspension and centrifugation in Krebs-Ringer-phosphate solution (16), and finally making up a cell suspension (hematocrit about 50) in the same solution. Hemolysates were prepared from washed erythrocytes (hematocrit about 50) by rapid freezing and thawing the solution three times as described previously (17).

Hemoglobin-free solutions were prepared by adding, in succession, to 150 ml of cold hemolysate contained in a 1000 ml flask, 300 ml of cold water, 96 ml of ethanol, and 60 ml of chloroform. The organic solvents were redistilled from reagent grade materials and stored at -20° before use. Immediately after the last addition, the mixture was shaken vigorously for 1 minute, transferred to precooled 100 ml glass centrifuge tubes, and centrifuged in the cold for 10 minutes at $2300 \times g$. The pink,³ aqueous top phase was withdrawn carefully with a syringe or suction pipette and dialyzed with stirring against a large volume of cold distilled water for 4 hours.

Methods—All manometric experiments were carried out in the standard Warburg apparatus at 37° with oxygen in the gas phase. The flasks were

³ When all of the endogenous hemoglobin has been denatured, the aqueous phase is almost colorless or light yellowish pink in appearance.

gassed from 3 to 5 minutes, followed by an equilibration period of 10 minutes before the stopcocks were closed. Oxygen uptakes were linear with time for at least 3 to 4 hours and linear with an amount of cells up to 1.5 ml. For both cell suspensions and hemolysates, the oxygen uptake values were recalculated, from the hematocrit data, on the basis of 1 ml. of packed cells (*i.e.* microliters of O_2 per hour per ml. of cells).

For measuring glucose oxidation in whole cells, the following assay procedure (System A) was used: 30 μ moles of glucose, 0.05 ml. of 1.0 per cent methylene blue, 1.0 ml. of washed red cell suspension (hematocrit about 50), and sufficient Krebs-Ringer-phosphate solution, or 0.1 M phosphate buffer, pH 7.5, to make 2.8 ml. The center well contained 0.2 ml. of 6 N NaOH.

TPNH oxidation in hemolysates was measured by the following assay procedure (System B): 30 μ moles of G-6-P, 0.50 ml. of G-6-P dehydrogenase (4 mg. per ml.), 0.25 μ mole of TPN, 30 μ moles of nicotinamide, 0.01 ml. of 1.0 per cent methylene blue, 100 μ moles of ethanol, 0.05 ml. of catalase (150 units per ml.), 1.0 ml. of hemolysate (derived from a cell suspension of hematocrit about 50), and sufficient 0.1 M phosphate buffer, pH 7.5, to make 2.8 ml. The center well contained 0.2 ml. of 6 N NaOH.

RESULTS AND DISCUSSION

Oxidation of Glucose by Intact Erythrocytes—In confirmation of previous results (10, 11) it was observed that intact erythrocytes oxidize glucose only to a small extent (0 to 5 μ l. per hour per ml. of cells) in the absence of methylene blue, but that the oxygen uptake is increased greatly (80 to 90 μ l. per hour per ml. of cells) when the dye is present at a final concentration of approximately 10^{-5} M. Other dyes and electron carriers, such as toluidine blue and thionine (12), or the bacterial pigment, toxoflavin (18), will replace methylene blue in this system. Rat liver extracts or purified diaphorase (11) are comparable to dyes in stimulating the oxidation of glucose. In view of these experiments, it seems evident that the oxidation of TPNH is linked to oxygen either through the addition of an electron carrier (codiaphorase) to saturate an apodiaphorase already existing in the erythrocyte or by the addition of a diaphorase. Kiese (12) and others (19, 20) have shown that the reduction of methemoglobin by TPNH is similarly dependent upon methylene blue, and have designated the electron-transferring enzyme, linking TPNH to oxygen or methemoglobin as *methemoglobin reductase*. The detailed purification and properties of this enzyme are described in Paper IV of this series (21).

Oxidation of Glucose by Hemolysates—When erythrocytes are hemolyzed, the ability to oxidize glucose is lost, even in the presence of added methylene blue (11). We have found that the oxidation can be restored, how-

ever, when the hemolysate is fortified with added Mg^{++} , ATP, and hexokinase, as illustrated in Table I. Apparently hemolysis results in some dispersion or destruction of each of these three components.

Oxidation of TPNH by Hemolysates—As an extension of the above experiments, it could be shown that the oxidation of TPNH, either added in

TABLE I
Glucose Oxidation by Hemolysates

Component omitted	O ₂ uptake
	μl per hr per ml cells
None (complete system)	270
Mg^{++}	190
Hexokinase	150
ATP	120
Hexokinase, ATP, Mg^{++}	12
MeB	0

System A was employed, except that 2.0 ml of hemolysate (equivalent to 1.0 ml of cells) were used and the following additions were made to the complete system: 1 μ mole of Mg^{++} , 5 μ moles of ATP, 0.05 ml of hexokinase (4 mg per ml), 1 μ mole of TPN, and 1 μ mole of nicotinamide.

TABLE II
TPNH Oxidation by Hemolysates

Component omitted	O ₂ uptake
	μl per hr per ml cells
None (complete system)	325
G-6-P dehydrogenase	307
G-6-P	42
MeB	12
TPN	0

System B was employed. Oxygen uptake values were corrected for the blank without G-6-P and methylene blue.

substrate amounts or generated continuously by the glucose-6-phosphate dehydrogenase system, required methylene blue. In Table II a component study is presented. An almost complete requirement was observed for both TPN and MeB as well as a strong requirement for G-6-P. Since the hemolysate was not dialyzed, it is probable that some endogenous G-6-P, or other substrate reducing TPN, was present in the preparation. There was only a small effect of added G-6-P dehydrogenase, in contrast to the hexokinase effect in Table I.

Oxygen Uptake in Aged Cells Rejuvenated by Adenosine—It has been shown previously that, when erythrocytes are stored for several weeks, the progressive loss of physiological viability is paralleled by a loss of endogenous organic phosphate esters, notably ATP and 2,3-diphosphoglycerate (22). This *in vitro* aging process can be retarded if the cells are stored in the presence of purine nucleosides such as adenosine or inosine. In the case of stored cells, a short term incubation with adenosine causes a marked rejuvenation.

In connection with these studies on the storage of erythrocytes, it was of interest to assay fresh cells and those aged *in vitro* (28 days) for the ability to oxidize glucose in the intact cell system (System A) and to oxidize

TABLE III
Effect of Adenosine on Oxygen Consumption in Fresh and Aged Cells

System	Blood sample	O ₂ uptake			
		Cells		Hemolysate	
		μl	$\text{per hr per ml cells}$	μl	$\text{per hr per ml cells}$
A	Fresh, control	88		300	
	“ treated with adenosine	115		315	
B	28 day storage, control	36		371	
	28 “ “ treated with adenosine	121		389	

A sample of blood was tested before and after 28 days of storage *in vitro*. In each case, an aliquot of the blood was incubated for 1 hour at 37° with adenosine (2100 μmoles of adenosine per 100 ml of cells). Washed cell suspensions and hemolysates then were prepared as described under “Experimental.” Oxygen uptake values were corrected for blanks without substrate.

TPNH in the hemolysate system (System B). As shown in Table III, glucose oxidation is somewhat improved even in the fresh sample and markedly improved in the 28 day sample after treatment of the respective cells with adenosine. Since there is essentially no decline of the enzyme, hexokinase, during storage (3, 22), the above results are consistent with the evidence that ATP declines during storage and is regenerated in the aged cell upon addition of adenosine. On the other hand, none of the components of the TPNH oxidation system declines upon storage, and, hence, there is no improvement after treatment with adenosine.

Partial Purification of TPNH Oxidation System—With use of System B, it was demonstrated that the level of the TPNH oxidase system is a linear function of time and of hemolysate concentration and that the level of TPNH oxidase is remarkably constant in different erythrocyte samples. The above findings provided adequate criteria to attempt the purification

of the enzyme system involved. It was found that there was no loss in activity when the hemolysates were freed from stroma by high speed centrifugation ($25,000 \times g$ for 30 minutes)⁴. The enzymatic activity was stable to dialysis, to repeated freezing and thawing of the hemolysate, to lyophilization, or to precipitation with 8 volumes of acetone in the cold. Acetone powders, prepared by precipitation from dialyzed hemolysates, could be stored in a desiccator for several months at -20° and they provided a stock starting material for further purification studies.

Previously, a partial isolation of the TPNH oxidase, or *methemoglobin reductase*, was achieved by Kiese by means of ammonium sulfate fractionation (12). The principal difficulty in purifying this enzyme from hemolysates is the removal of hemoglobin, which comprises about 95 per cent of the total soluble protein (2). From blood collected in oxalate, Foulkes (23) was able to crystallize a considerable amount of hemoglobin as the carbon monoxide complex, but in our hands this proved to be impossible with hemolysates prepared from blood collected in ACD. Similarly, it is of interest to note that hemoglobin can be crystallized by Drabkin's method (24) from blood collected in oxalate, but not from blood collected in ACD.⁵

Efforts to free the TPNH oxidase from hemoglobin by conventional means were largely unsuccessful, for, although the enzyme could be concentrated by precipitation with ammonium sulfate at 60 per cent saturation or with ethanol at 50 per cent saturation, the activity was found also in other fractions, and all fractions were still contaminated heavily with hemoglobin. In addition, the partially purified fractions were extremely unstable, whether stored at 5° or frozen. This was due, presumably, to the presence of a destructive protein, since, if the hemolysates were treated by Kiese's method (12) (*i.e.* adjusted to 1 M in sodium phosphate and the slight precipitate removed by centrifugation), the subsequent fractions obtained by ammonium sulfate precipitation were much more stable to storage.

Treatment of hemolysates with ethanol-chloroform at low temperatures,⁶ a technique previously employed for the isolation of carbonic anhydrase (26) and peptidases (27) from erythrocytes, resulted in the complete denaturation of hemoglobin, but left the TPNH oxidase unharmed. With use of System B, it was found that more than 70 per cent of the original activity in hemolysates was recovered in the aqueous phase after the above treatment. Since the solution containing the enzyme was essentially color-

⁴ Specialized Instruments Corporation ultracentrifuge, rotor No. 40.

⁵ Unpublished observations of this laboratory.

⁶ Essentially the same results can be obtained by shaking the hemolysate with *n*-butanol at low temperatures, according to the procedure of Morton (25).

less, TPNH oxidase could now be estimated spectrophotometrically. The details of this assay, and the further purification and properties of the enzyme, are described in Paper IV (21).

The authors are indebted to Mrs. Ruth Wade Caffrey for her assistance on portions of this problem.

SUMMARY

1. Intact erythrocytes oxidize glucose only in the presence of an added diaphorase (tissue extract) or an added codiaphorase (methylene blue). Upon hemolysis, an additional requirement for added hexokinase, Mg^{++} , and adenosine triphosphate becomes evident.

2. Oxidation of glucose proceeds via the "phosphogluconate oxidation" pathway. The oxidation of glucose 6-phosphate to 6-phosphogluconate is linked to oxygen or methemoglobin through triphosphopyridine nucleotide and an electron transport system, probably identical with Kiese's *methemoglobin reductase*. The partial purification of this enzyme has been achieved by removal of hemoglobin with an ethanol-chloroform treatment.

3. Manometric assay systems are described for the oxidation of glucose in whole cells and for the oxidation of reduced triphosphopyridine nucleotide in hemolysates. Stored red cells aged *in vitro* and rejuvenated with adenosine may be assayed conveniently by these systems, and the relation between adenosine triphosphate loss and the storage lesion is further established.

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ERYTHROCYTE METABOLISM

IV ISOLATION AND PROPERTIES OF METHEMOGLOBIN REDUCTASE*

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In mature, non-nucleated erythrocytes the breakdown of glucose to pentose, via the "phosphogluconate oxidation" pathway, includes an oxidative step, whereby glucose 6-phosphate is converted to 6-phosphogluconate in the presence of TPN¹ and the appropriate dehydrogenase (2). It has been shown previously by other investigators (2-4), and amplified in Paper III of this series (5), that TPNH, formed in the primary dehydrogenation, is linked to oxygen through a TPNH oxidase. The isolated enzyme requires an added electron carrier such as methylene blue for its activity.

A similar situation obtains in the reduction of methemoglobin by the erythrocyte. The low (about 1 per cent (6)) steady state level of methemoglobin in the cell indicates that the slow, continuous chemical oxidation of hemoglobin by molecular oxygen is nearly compensated for by the enzymatic reduction of methemoglobin. Kiese (7) has partially purified the enzyme, methemoglobin reductase, and has shown that TPNH is the substrate for the enzyme and that methylene blue serves as a cofactor. Evidence has been presented also for the existence of a similar, but separate, system requiring DPNH. In accordance with varying sources of evidence Kiese has suggested that the TPNH oxidase and the TPNH methemoglobin reductase are the same enzyme.

The present communication describes the isolation, in highly purified form, and the properties of the methemoglobin reductase from human erythrocytes. Observations are presented concerning TPNH and DPNH

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¹ The following abbreviations are used: DPN and TPN, di- and triphosphopyridine nucleotides, DPNH and TPNH, reduced DPN and TPN, Hb, reduced hemoglobin, HbO₂, oxyhemoglobin, MHb, methemoglobin, MeB, methylene blue.

as substrates, and oxygen, methemoglobin, and cytochrome *c* as terminal electron acceptors

EXPERIMENTAL

Materials—Crystalline hemoglobin was prepared from human blood² by the method of Drabkin (8), and methemoglobin by the oxidation of hemoglobin with NaNO_2 or H_2O_2 (9). Isocitric dehydrogenase was isolated from pig heart (10). *dl*-Isocitric acid lactone was obtained from the California Foundation for Biochemical Research, crystalline alcohol dehydrogenase was a product of the Mann Research Laboratories, Inc., TPNH, DPNH, and cytochrome *c* were obtained from the Sigma Chemical Company, 1,10-*o*-phenanthroline from the G. Fredrick Smith Chemical Company, and iron-specific Versene from the Bersworth Chemical Company. Other materials were obtained as described in Paper III (5).

Methods—Protein concentrations were determined by the biuret method (11) with bovine serum albumin as the standard. Pyridine hemochromogen determinations were carried out as described by Basford *et al.* (12) with a molar extinction coefficient, ϵ , equal to 34.7×10^6 sq cm per mole (13) at 557 $m\mu$ for the hemochromogen of iron protoporphyrin. Total iron was estimated by the method of Sandell (14).

Spectrophotometric measurements were made in 1 cm cuvettes in the Beckman spectrophotometer, model DU. Absorption spectra were obtained with a Cary recording spectrophotometer, model 11M.

Assay of the enzyme as a TPNH oxidase (*i.e.* with oxygen as the terminal electron acceptor) was carried out as follows. The experimental cuvette contained 0.15 μmole of TPNH, 100 μmoles of phosphate buffer,³ pH 7.5, 0.2 ml of enzyme, and water to make 3.0 ml. The blank cuvette was identical except for the omission of TPNH. After a stable, initial $\log I_0/I$ value at 340 $m\mu$ had been obtained, 0.01 ml of 0.1 per cent methylene blue (0.027 μmole) was added to each cuvette at zero time and the reaction was followed by the decrease in light absorption over a 10 minute period. The reaction velocity was corrected for the slow, chemical oxidation of TPNH by the dye ($\Delta_{10} = 0.022$) in a blank cuvette from which the enzyme was omitted.

The reaction velocity is linear with time and can be expressed as a Δ_{10} value, *i.e.* the change in $\log I_0/I$ at 340 $m\mu$ over the 10 minute period, under conditions of enzyme concentration whereby Δ_{10} is less than 0.060. 1

² We are indebted to Dr. Richard Czajkowski and Mr. Soren Jule of the King County Blood Bank, Seattle, for collecting and making available the blood for this study.

³ At the optimal pH for the enzyme (pH 7.5), there was no diminution of activity when tris(hydroxymethyl)aminomethane or Veronal replaced phosphate as the buffer.

unit of enzyme is defined as that amount which causes $\Delta_{10} = 0.010$ under these conditions. Specific activity is defined as units of enzyme per mg of protein. DPNH oxidase activity is determined by the above spectrophotometric system with an equivalent amount of DPNH replacing the TPNH.

In order to study the enzyme as a methemoglobin reductase or cytochrome *c* reductase, the spectrophotometric assay system was modified to include 0.12 μ mole of MHb or 0.10 to 0.20 μ mole of cytochrome *c*. The methemoglobin reductase reaction was followed by the disappearance of the MHb band at 630 $m\mu$, or the appearance of the HbO₂ band at 575 $m\mu$. The reaction with cytochrome *c* was followed by the appearance of the reduced cytochrome *c* band at 550 $m\mu$.

RESULTS AND DISCUSSION

Purification of Enzyme

Step 1 Removal of Hemoglobin from Hemolysates—Fresh, human erythrocytes (125 ml of packed cells) were washed three times by suspension and centrifugation⁴ in cold, 0.9 per cent NaCl solution,⁵ diluted to the original volume (about 300 ml) with saline, and hemolyzed by repeated freezing and thawing. As described in Paper III (5), the hemolysate was treated at low temperature with ethanol and chloroform to remove hemoglobin and was dialyzed.

Step 2 Fractionation at pH 5.4—The dialyzed enzyme from Step 1 (865 ml) was lyophilized to dryness in order to remove traces of organic solvents and redissolved in 80 ml of water. The dilute solution was allowed to stand overnight at 5°, and any precipitate which formed was removed by centrifugation. The solution was then adjusted to pH 5.4 with 10 per cent acetic acid, the precipitate removed by centrifugation, and the supernatant solution readjusted to pH 7.0 with 1 *N* sodium hydroxide. The amount of inert protein removed by the aging step varied somewhat with different preparations, however, the data given in Table I are representative of a number of individual preparations.

Step 3 Ammonium Sulfate Fractionation—To the solution from Step 2 (80 ml), immersed in an ice bath, were added slowly with stirring 80 ml of saturated ammonium sulfate solution neutralized to pH 7.0 with concentrated ammonium hydroxide. The precipitate (0 to 50 per cent fraction)

⁴ All centrifugations were carried out at 4° for 15 minutes at $2300 \times g$ in the International refrigerated centrifuge, model PR-1.

⁵ It has been found that cells washed thoroughly with 0.9 per cent NaCl before hemolysis yield enzyme preparations at the end of Step 1 which have a 5- to 10-fold higher specific activity compared to cells washed with Krebs-Ringer-phosphate solution.

was removed by centrifugation and discarded. Successive addition of 40, 67, and 135 ml of ammonium sulfate to the 0 to 50 per cent supernatant fraction yielded the 50 to 60, 60 to 70, and 70 to 80 per cent precipitates, which were removed by centrifugation, dissolved in 40 ml of water, and dialyzed overnight against water. After dialysis, each of the above three fractions was centrifuged to remove any precipitate and assayed for activity. The highest specific activity and most of the total activity were found in the 60 to 70 per cent fraction, which was used for all the experiments reported in this paper. The 50 to 60 or 70 to 80 per cent fractions could be refractionated with ammonium sulfate to obtain additional quantities of the enzyme.

For a number of preparations the specific activity after ammonium sulfate fractionation ranged between 68 and 72. No further purification

TABLE I
Purification Procedure

Fraction	Total volume	Total protein	Total units	Specific activity	Recovery of units
	<i>ml</i>	<i>mg</i>		<i>units per mg</i>	<i>per cent</i>
Hemolysate	300	42,000*	16,760	0.4	100
Ethanol-chloroform fractionation	865	490	12,300	25	73
Fractionation, pH 5.4	80	220	8,650	39	52
(NH ₄) ₂ SO ₄ fractionation, 60-70%	40	80	5,760	72	34

* Based upon hemoglobin determination

could be achieved by several other methods of protein fractionation or by a repetition of the above methods.

The enzyme may be concentrated at any stage of purification by precipitation with neutral ammonium sulfate at 75 per cent saturation, by precipitation with acetone, or by lyophilization. The enzyme was not adsorbed on to calcium phosphate or zinc hydroxide gel, and these treatments could be used to remove extraneous protein.

Table I summarizes the specific activities and recovery data at various stages for a typical preparation. At the final stage the enzyme was purified approximately 180-fold, based upon the hemolysate, and recovered in a 34 per cent over-all yield.

In hemolysates the enzymatic activity was decreased about 50 per cent during a 24 hour storage period at 5° or frozen at -20°. However, after treatment with ethanol-chloroform, and at all subsequent stages, the enzyme retained essentially full activity upon storage in the frozen state for periods up to 1 month.

Existence of Iron Porphyrin Prosthetic Group—After treatment of the

hemolysate with chloroform-ethanol, the enzyme in the aqueous phase was light yellow-pink in color. Upon subsequent purification the preparation assumed the characteristic red-brown appearance of iron porphyrin enzymes. The spectrum⁶ of the highly purified enzyme (specific activity = 70) at pH 7.5, shown in Fig. 1, is clearly that of a heme protein, since, in addition to the absorption band at 278 $m\mu$ due to the protein moiety, there is a large Soret peak at 406 $m\mu$ and several smaller peaks in the spectral region of 500 to 650 $m\mu$. Upon treatment of the enzyme with suitable reagents, three separate states of the heme moiety, analogous to those of hemoglobin, can be recognized by their spectral characteristics. After treat-

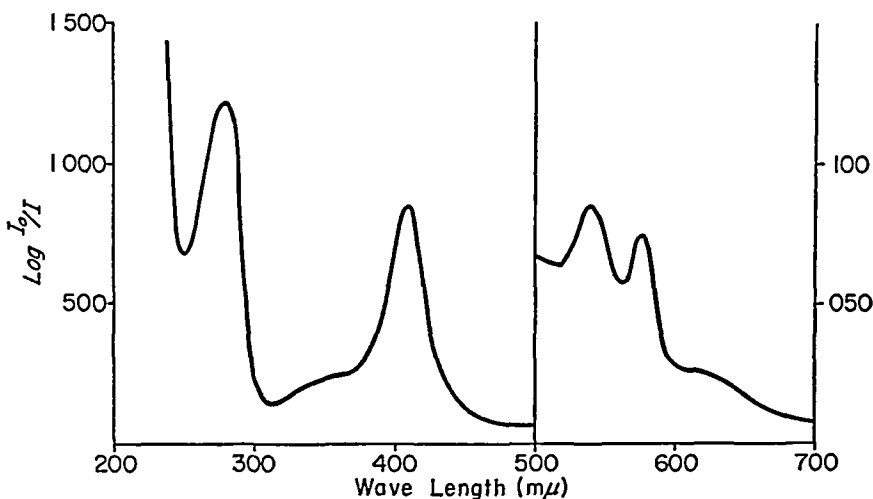


Fig. 1. Absorption spectrum of purified enzyme. The spectrum was determined with use of the Cary recording spectrophotometer on an enzyme of specific activity = 70 at pH 7.5. The protein concentration was 1.0 mg per ml.

ment of the enzyme with ferricyanide, the oxidized form displays peaks at 403, 500, and 630 $m\mu$, after treatment with hydrosulfite, the reduced enzyme has peaks at 431 and 555 $m\mu$, after reduction of the enzyme with TPNH in the presence of air, the reduced-oxygenated complex is formed, characterized by peaks at 415, 540, and 577 $m\mu$. The details of these spectra will be published separately.⁷

As seen in Fig. 1, the isolated enzyme is evidently a mixture, inasmuch as the heme is distributed between the oxidized and reduced-oxygenated states. Since the molar extinction coefficient for the Soret peak of these

⁶ In the early stages of purification (*i.e.* after the "ethanol-chloroform step") the enzyme has an additional, broad absorption band centered at 260 $m\mu$, but this is lost, especially after ammonium sulfate fractionation.

⁷ Caffrey, R. W., Talbert, P. T., and Huennekens, F. M., in preparation.

two states is almost identical,⁸ the ratio of the protein peak at 278 $m\mu$ to the composite Soret peak, located between 405 and 408 $m\mu$, may be used as a measure of the purification of the enzyme. The ratio is 1.45 for the purified enzyme.

That the iron porphyrin is associated with the enzyme itself and is not due to traces of contaminating hemoglobin is substantiated by several observations. First, the absorption maxima of the various states of the enzyme are slightly, but significantly, different from those of hemoglobin. Second, repeated treatment of the enzyme with ethanol-chloroform under conditions identical with those of Step 1 in the purification procedure, whereby hemoglobin is denatured, does not lead to any further decrease in color or to further denaturation. Third, the ratio of enzymatic activity to the light absorption due to the porphyrin moiety is constant after the first stage during purification. Fourth, the enzyme is eluted from ion exchange columns under conditions whereby the multiple forms of hemoglobin (16) are still adsorbed.⁷ Fifth, crystalline hemoglobin itself displays no activity as a methemoglobin reductase. Finally, under anaerobic conditions and with substrate amounts of enzyme, the iron porphyrin moiety can be reduced quantitatively by substrate amounts of reduced methylene blue or by substrate amounts of TPNH in the presence of catalytic amounts of methylene blue. These results will be reported in a subsequent communication.⁷

It is proposed, then, that the iron porphyrin group is a functional part of the enzyme, and that it does not represent traces of contaminating hemoglobin, although the iron porphyrin moiety of the two proteins may be identical.

Molecular Weight Determination from Total Iron and Hemochromogen Analyses—Two separate aliquots of the purified enzyme were converted to the pyridine hemochromogen. The absorption maxima were at 418, 522, and 553 $m\mu$. From the optical density values at 553 $m\mu$, the heme content of the enzyme was calculated to be 5.47 and 5.51 $\times 10^{-6}$ mmole per mg of protein. Assuming 1 heme per mole, this would correspond to a minimal molecular weight of 182,000. Total iron determinations, with cytochrome c as an internal control for the analytical method, gave an average value of 5.31 $\times 10^{-6}$ mmole per mg of protein, or a molecular weight of 188,000. The actual molecular weight of the enzyme may be lowered slightly by a factor representing the absolute purity of the enzyme.

Spectrophotometric Demonstration of Activity—At all stages of purification

⁸ A solution of the enzyme (specific activity = 72) containing 1.63 mg per ml of total protein had an optical density of 1.050 at 406 $m\mu$. Assuming a molecular weight of 1.85×10^4 , this would correspond to $\epsilon = 119 \times 10^6$ sq cm per mole for the Soret peak. The ϵ values for the Soret peaks of HbO₂ and MHb are each approximately 130×10^6 sq cm per mole (15).

beyond the hemolysate, the enzyme can be assayed most conveniently by following the disappearance of TPNH spectrophotometrically at $340\text{ m}\mu$. As shown in Fig 2, TPNH was oxidized slowly by endogenous oxygen in the presence of enzyme alone, but the rate was increased markedly upon the addition of the methylene blue. After the TPNH had disappeared completely, the reaction was stopped by heat denaturation, and the super-

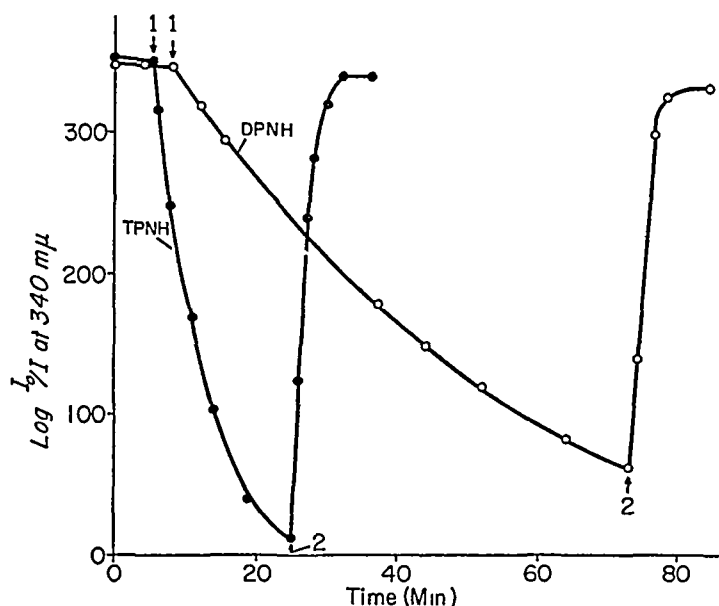


Fig 2 Spectrophotometric demonstration of TPNH and DPNH oxidase ●, TPNH, ○, DPNH. Assay system as described under "Experimental," except that the TPNH or DPNH was increased to $0.17\text{ }\mu\text{mole}$ and the amount of enzyme was increased to 20 units. Methylene blue was added at Arrow 1. Values are not corrected for the blank without enzyme which amounted to a Δ_{10} value of 0.022. At Arrow 2, the reaction was stopped by heating to 100° for 5 minutes, the denatured protein removed by centrifugation, and the supernatant fluid returned to the cuvette. In the experiment with TPNH, $6\text{ }\mu\text{moles}$ of isocitrate, $2\text{ }\mu\text{moles}$ of MgCl_2 , and 0.02 ml of isocitric dehydrogenase were added and the light absorption was followed again. In the experiment with DPNH, $200\text{ }\mu\text{moles}$ of Tris buffer, pH 9.5, $100\text{ }\mu\text{moles}$ of ethanol, and 0.01 ml of alcohol dehydrogenase were added.

natant fluid, after a brief centrifugation, was analyzed for TPN with the components of the glucose-6-phosphate dehydrogenase system. The light absorption returned nearly to its initial value, showing that the initial reaction was that of TPNH oxidation rather than TPNH destruction.

The DPNH activity of the enzyme was demonstrated by a parallel experiment (see also Fig 2) in which conversion of DPNH to DPN in the initial reaction was shown by adding the components of the alcohol dehydrogenase system.

Ratio of Activity with TPNH and DPNH—Previous work had indicated

the existence of two separate enzymes for the reduction of MHb, one specific for TPNH and the other for DPNH. For example, Kiese (7) demonstrated in hemolysates that glucose and lactate were additive in their ability to reduce MHb and that, when each of these substrates was employed, a different K_m value was obtained for the cofactor, methylene blue.

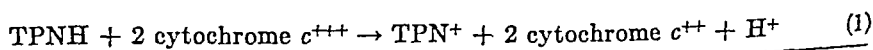
With the enzyme at the highest stage of purification, the ratio of TPNH to DPNH activity was 5.4 when the assays were performed under parallel conditions. This ratio is not appreciably different from that at Step 1, where the value was approximately 3.5. In the latter case, the ratio is less reliable, owing to the relatively large amount of protein required for the assay. These data suggest that the present enzyme, as isolated, is specific for TPNH, but that it has some activity with DPNH. There may exist, in addition, a DPN-specific reductase which is destroyed during the above isolation procedure.

Inhibitors—The enzyme is inhibited (a) approximately 25 per cent by Ca^{++} , Ba^{++} , Mn^{++} , sulfate, and pyrophosphate, each at a final concentration of 10^{-2} M, (b) 40 per cent by Zn^{++} or atabrin at 10^{-3} M, (c) 20 per cent by *p*-chloromercuribenzoate at 10^{-5} M, and (d) 50 per cent by Hg^{++} at 10^{-5} M. The enzyme is not inhibited appreciably by riboflavin, riboflavin phosphate, flavin adenine dinucleotide, 2,4-dinitrophenol, gramicidin, 8-hydroxyquinoline, isonicotinic hydrazide, arsenite, arsenate, iodoacetate, iodosobenzoate, or Versenate, each at a final concentration of 10^{-2} M. The pattern of inhibition for this enzyme is similar to that observed previously for the DPNH oxidase isolated from pig heart (17).

Terminal Electron Acceptors—In Paper III (5) and in the present investigation it has been shown that oxygen serves as a terminal electron acceptor in the presence of catalytic amounts of methylene blue. Under anaerobic conditions, *i.e.* in evacuated cuvettes or in Thunberg tubes, methylene blue or 2,6-dichlorophenol-indophenol may be used as an acceptor.

Kiese has presented evidence that either methemoglobin or oxygen can be used as terminal electron acceptors for the enzyme and that they are competitive with each other. Using the purified enzyme, we have confirmed his findings and have found that cytochrome *c*⁹ may be used also as a terminal acceptor. A summary of comparative rates of reduction for the three acceptors, oxygen, MHb, and cytochrome *c*, is presented in Table II.

For either cytochrome *c* or MHb, the stoichiometry is as follows



⁹ There is no crossed specificity in this reaction, since methemoglobin will not replace cytochrome *c* in the DPNH cytochrome *c* reductase from pig heart (18) or the TPNH cytochrome *c* reductase from yeast (19). Altman (20) has found, however, that a preparation from brewers' yeast contains MHb reductase activity.

The reduction of these acceptors was followed by means of the optical density changes at 550 $m\mu$ for cytochrome *c* and 630 $m\mu$ or 575 $m\mu$ for MHb. The initial rates, linear over a 0 to 5 minute interval, were 39.6×10^{-7} mmoles per ml per minute for cytochrome *c* and 3.6×10^{-7} for MHb. According to Equation 1, these values would correspond to rates of TPNH oxidation of 19.8×10^{-7} and 1.8×10^{-7} , respectively. Since the actual rates of TPNH oxidation, as measured by changes in optical density at

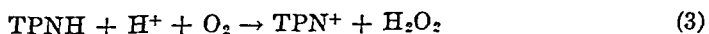
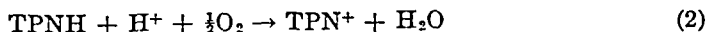
TABLE II
Terminal Electron Acceptors

Acceptor	Rate of TPNH disappearance, mmoles per ml per min $\times 10^7$		
	Observed	Due to porphyrin	Due to O ₂
Cytochrome <i>c</i>	22.5	19.8	2.7
Oxygen	12.5		12.5
Methemoglobin	10.0	1.8	8.2

The standard assay system was used as described under "Experimental," except that 0.3 μ mole of TPNH was used. Successive spectra at given time intervals were run on the Cary recording spectrophotometer. For oxygen, the reaction was followed at 340 $m\mu$, with $\Delta\epsilon$ of 6.22×10^6 sq cm per mole being used for the difference between TPNH and TPN. For cytochrome *c*, the reaction was followed at 550 $m\mu$ by use of $\Delta\epsilon = 18.5 \times 10^6$ (21) for the difference between oxidized and reduced cytochrome *c*, and at 340 $m\mu$ for TPNH ($\Delta\epsilon = 0$ for oxidized and reduced cytochrome *c* at 340 $m\mu$). For MHb, the reaction was followed at 630 $m\mu$ ($\Delta\epsilon = 4.0 \times 10^6$ (15)), at 575 $m\mu$ ($\Delta\epsilon = 14.0 \times 10^6$ (15)), and at 340 $m\mu$ ($\Delta\epsilon = 7.0 \times 10^6$ for MHb and HbO₂ at 340 $m\mu$). The rate of reduction of MHb calculated from the optical density changes at 630 $m\mu$ was 3.0×10^{-7} , and 3.6×10^{-7} , the changes at 575 $m\mu$ being utilized. The latter value was used, since the $\Delta\epsilon$ at 575 $m\mu$ is larger. All the rates were taken over an initial period (0 to 5 minutes) and were corrected for blanks without enzyme.

340 $m\mu$, were 22.5×10^{-7} and 10.0×10^{-7} , it is apparent that the differences, 2.7×10^{-7} and 8.2×10^{-7} , represent the oxidation of TPNH due to oxygen, acting in competition with the porphyrins.

When oxygen is used alone as the acceptor, the rate of TPNH oxidation is 12.5×10^{-7} mmole per ml per minute. From this value the rate of oxygen reduction would be either 6.3×10^{-7} or 12.5×10^{-7} , depending upon whether Equation 2 or 3 is operative.

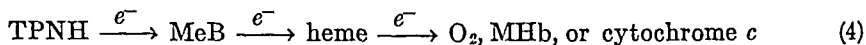


At present, it has not been possible to determine conclusively whether H_2O or H_2O_2 is the product of oxygen reduction in this system¹⁰

It is apparent that cytochrome *c* is a preferred acceptor to oxygen, for the initial concentration of the heme compound was approximately 3×10^{-5} M, as compared to the oxygen concentration of approximately 2.4×10^{-4} M (22)

The favored utilization of cytochrome *c* by the enzyme is somewhat surprising, inasmuch as the mature erythrocyte contains no endogenous cytochrome *c*. It is possible that, in the nucleated or immature stage, the erythrocyte uses the enzymes as a cytochrome reductase, but that, upon maturation and the concomitant reversion to a more anaerobic type of metabolism, the enzyme functions only as a methemoglobin reductase.

Electron Transport Scheme—From the evidence presented above, it seems permissible to formulate the electron transport scheme of the enzyme as follows



The enzyme is thus considered to have two prosthetic groups: (1) an unknown carrier, which is detached during purification and must be substituted for by methylene blue or other autooxidizable dyes, and (2) a tightly bound iron porphyrin moiety. Under anaerobic conditions it can be shown⁷ that TPNH reacts first with the dye, that the reduced dye interacts readily with the iron porphyrin, and that the iron porphyrin, in turn, reduces molecular oxygen. Under aerobic conditions it is possible that the reduced dye would interact directly with oxygen, thus by-passing the porphyrin component. However, we have been unable to demonstrate the formation of hydrogen peroxide under these conditions¹⁰

Role of Methylene Blue and Other Carriers—As shown in this investigation and previously (5), the enzyme, as isolated from human erythrocytes, has an absolute requirement for methylene blue, or other autooxidizable dyes, as an electron carrier. Toxoflavin, a pigment elaborated by *Pseudomonas cocovenen* (*Bacterium bongkrek*), also stimulates the enzyme (23). In the search for the physiological electron carrier, a great variety of known coenzymes and metal ions were tried, including riboflavin, flavin mononucleotide, flavin adenine dinucleotide,¹¹ reduced glutathione, ascorbic acid,

¹⁰ When excess catalase and ethanol were added to the manometric system, there was observed no doubling of the oxygen uptake, as noted previously with the DPNH oxidase from pig heart (17). This fact would suggest that oxygen is being reduced to water rather than to hydrogen peroxide in this system.

¹¹ The purified enzyme contains no flavin, as evidenced by the absorption spectrum (see Fig. 1) or by paper chromatographic examination (24) of the supernatant fluid of heat- and trichloroacetic acid-denatured preparations.

and eigothioneine. All were without effect. Recently, we have found that the methemoglobin reductase, isolated from beef erythrocytes, does not require methylene blue for activity. When the beef blood enzyme is boiled, a material is obtained which activates the human reductase.⁷ The purification and properties of this natural cofactor are currently under investigation.

It is of interest to recall that the disease, congenital methemoglobinemia, which is characterized by steady state levels of MHb in the erythrocytes as high as 10 to 30 per cent, has been treated successfully by periodic infusion of massive amounts of methylene blue (25).

The authors are indebted to Dr. Preston Talbert for his contribution to this problem and to Dr. Clement Finch for many helpful discussions on the clinical aspects of methemoglobinemia.

SUMMARY

1. Reduced triphosphopyridine nucleotide oxidase, or methemoglobin reductase, has been isolated from hemolysates of human, mature erythrocytes by means of ethanol-chloroform treatment, precipitation of inert proteins at pH 5.4, and ammonium sulfate fractionation.

2. The enzyme is a hemoprotein with a molecular weight of approximately 185,000, based upon pyridine hemochromogen and total iron determinations.

3. The enzyme oxidizes reduced triphosphopyridine nucleotide, and to a lesser extent reduced diphosphopyridine nucleotide, with oxygen, methemoglobin, or cytochrome *c* as the terminal electron acceptor. Methylene blue is required as an electron carrier.

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ADRENOCORTICAL STEROID C-20-KETO REDUCTASE*

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Previous work from this laboratory established that whole homogenates of rat liver actively alter the characteristic 17,21-dihydroxy-20-ketone side chain of adrenocortical steroids (2). The enzyme was found in both the microsome and supernatant fractions obtained by conventional¹ differential centrifugation (3). The rate of the reaction was markedly accelerated upon the addition of cosubstrates (citrate, isocitrate, G-6-P²) oxidized by TPN-dependent dehydrogenases (3). It has also been shown by product identification that the degradation reaction involves a reduction of the C-20 ketone to the corresponding C-20 alcohol (4). There seems to be little doubt that the C-20-keto reductase³ described here for rat liver is identical with the enzyme system described for rat liver by Hubener *et al* (7), Forchielli *et al* (8), and Eisenstein (9), and is similar to that described for hog liver by Caspi *et al* (10) and for rabbit liver by Taylor (5). This report extends our earlier studies on the intracellular localization of the C-20-keto reductase reaction. Data on the pyridine nucleotide requirement of the C-20 keto reduction are presented, as well as observations on the stability of the enzyme and the relative inactivity of the C-20-keto reductase system against steroids lacking the 17 α -hydroxyl group. Previous observations by others on the liver metabolism of adrenocortical steroids are discussed in the light of the data presented in this report.

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¹ The maximal gravitational field available in the preliminary work was 20,000 $\times g$.

² Abbreviations used are as follows: G-6-P, glucose 6-phosphate, TPN, triphosphopyridine nucleotide, DPN, diphosphopyridine nucleotide, DPNH and TPNH, the corresponding reduced cofactors, Tris, tris(hydroxymethyl)aminomethane, RNA, ribonucleic acid, DOC, deoxycorticosterone, RNase, ribonuclease.

³ The term C-20-keto reductase is suggested for the system reducing the C-20 ketone of adrenocortical and possibly other steroids (5). The justification for this term, apart from its obvious simplicity and suggestiveness, lies in the fact that a major metabolic pathway of the C-20 ketone involves reduction to the alcohol (6).

EXPERIMENTAL

Male rats (The Holtzman Company, Madison 4, Wisconsin), 150 to 300 gm fed *ad libitum*, were used. The animals were killed by cervical section and exsanguinated. The liver was removed and placed in ice-cold 0.25 M sucrose which was 0.04 M with respect to nicotinamide. This medium was used throughout all differential centrifugation steps which were at 0–2°.

Homogenization and Differential Centrifugation—Homogenization was carried out with an all-Lucite homogenizer of the Potter-Elvehjem type. The centrifugation techniques employed were adapted to the nature of the experiments. For the study of the intracellular localization of the C-20-keto reductase, a typical sedimentation and washing routine was employed. 10 per cent whole homogenates of rat liver were centrifuged (International table model centrifuge, head No. 213 or 215) twice for 12 minutes at $600 \times g$ to remove the whole cells and nuclei. The nuclear sediment was washed twice. The combined homogenate and washings were sedimented at $6000 \times g$ for 15 minutes (American Instrument Company, Inc., high speed angle centrifuge, equipped with refrigeration, tube angle 32°). These centrifugation conditions sediment all mitochondria and some microsomal material (11). Upon resuspension and resedimentation (two cycles), some microsomal material appears as a loose fluffy layer, which was removed and added to the S_2 fraction.⁴ For experiments in which the distribution of the C-20-keto reductase was studied in the S_2 fraction, or for obtaining microsomes rapidly for routine use, whole homogenates were centrifuged at $6000 \times g$ to remove nuclei and mitochondria together, and the sediment was washed once. The resulting S_2 fraction, including any loose fluffy material, was then subjected immediately to enzyme study. The Spinco preparative ultracentrifuge, model L, was used to sediment microsomes.

Materials—Glucose-6-phosphate dehydrogenase, TPN, DPN, and DPNH were products of the Sigma Chemical Company. TPNH was prepared from TPN by a slight modification of the hydrosulfite method of Gutchko and Stewart (12). A molar absorptivity index of 6.22×10^3 liters per mole per cm (13) was used to calculate TPNH and DPNH concentrations from readings at 340 m μ in the Beckman DU spectrophotometer. Crystalline alcohol dehydrogenase was obtained from the Nutritional Biochemicals Corporation. The isocitric dehydrogenase preparation was an extract (0.1 M potassium phosphate buffer, pH 7.4) of pig heart acetone powder. The undiluted phosphate buffer extract had an activity

⁴ Differential centrifugation terminology used is as follows. S_2 fraction, nuclei- and mitochondria-free supernatant fraction, S_3 fraction, final particle-free supernatant fraction. An equivalent mg (eq mg) of any fraction is the total amount of that fraction obtained from 1 mg of wet weight of whole liver.

of 2480 units per ml, assayed according to Grafflin and Ochoa (14) The cortisone used was a product of The Glidden Company, the corticosterone was a gift of Dr A Zaffaroni, Syntex Research Laboratories, Mexico City, the deoxycorticosterone was a gift from Dr E Myles Glenn, The Upjohn Company, Kalamazoo

Analytical—The course of the steroid reduction reaction was followed by substrate disappearance, the disappearance of the α -ketol side chain of DOC and corticosterone was followed with the blue tetrazolium reaction (15) Disappearance of the 17,21-dihydroxy-20-ketone side chain of cortisone was traced with the Porter-Silber reaction (16) and with blue tetrazolium The total reaction volume was 5 ml The reaction was stopped by the addition of 20 ml of CHCl_3 Extraction was carried out by mechanical shaking The CHCl_3 extract was washed according to Glenn and Recknagel (2) Assays for unchanged steroid substrate were carried out on aliquots of the CHCl_3 extract, after removal of the CHCl_3 by evaporation under a stream of clean air Tissue blank values were rarely above 0.040 optical density unit in any of the analytical procedures and were usually 0.025 or lower The CHCl_3 extraction procedure efficiently removes unchanged steroids In one series of fifteen experiments, the recovery of cortisone from control tubes as assayed spectrophotometrically at 240 $\text{m}\mu$ was 97.9 per cent, with a standard deviation of 5.18 per cent In a parallel series of twenty experiments, the recovery of cortisone assayed according to Porter and Silber (16) was 98.0 per cent, with a standard deviation of 3.6 per cent

RESULTS AND DISCUSSION

Pyridine Nucleotide Dependence of C-20-Keto Reductase Reaction—Experimental investigation of the intracellular localization of the C-20-keto reductase system and further efforts to purify the enzyme clearly require a supply of the necessary cofactors for the reaction It is for this reason that the question of the source of the hydrogen in the reduction reaction, *i.e.* the possibility that a reduced pyridine nucleotide may function as a specific cofactor, is decisive at the present time in the further study of this problem The pyridine nucleotide requirement of the C-20-keto reductase reaction was first suggested in view of the interpretation of the "dilution effect" data of Recknagel and Glenn (3) It was observed that the C-20-keto reductase activity of relatively high concentrations of rat liver S_2 fractions was proportional to enzyme concentration but that the activity of the system upon dilution decreased far more than would be expected from the dilution factor alone This dilution effect is characteristic of complex cofactor-requiring or multienzyme systems and suggests that a necessary factor or factors are present in sufficiently high concentration

when large amounts of the crude enzyme are added but is diluted out when lesser amounts of enzyme are used. The fact that the dilution effect was overcome upon addition of citrate, isocitrate, or G-6-P suggested that the system required TPNH as hydrogen donor, since these substrates are oxidized by TPN-dependent dehydrogenases. Further indirect evidence bearing on the pyridine nucleotide requirement of the C-20-keto reductase reaction may be derived from observations on the apparent instability of the enzyme. It was observed earlier by Glenn and Recknagel (2), and more recently by Hubener *et al.* (7), that the C-20-keto reductase activity of the microsomes decreases during differential centrifugation procedures. The fact that the C-20-keto reductase system has been shown to be a relatively stable enzyme (see below), provided that the assay is carried out in the presence of a TPNH-generating system, clearly indicates the pyridine nucleotide requirement of the system. The enhancement in activity on recombination of microsomes and supernatant fraction reported earlier by Recknagel and Glenn (3), and more recently by Hubener *et al.* (7), appears to be due to the presence in the supernatant fraction of the components of an endogenous TPNH-generating system. The conclusion of Hubener *et al.* (7) that the C-20-keto reductase reaction is not TPNH-dependent was based on an experiment in which the enzyme preparation was the supernatant fraction of a phosphate buffer, KCl homogenate of rat liver after centrifugation at $17,500 \times g$. This procedure would effectively remove a large fraction of the active enzyme which can be sedimented in part at $20,000 \times g$, even from 0.25 M sucrose homogenates which are known not to have the agglutinating effects on cell particulates as do media of high ionic strength (17). Hubener *et al.* (7) based their conclusion relevant to the non-dependence of the C-20 keto reduction on TPNH on the fact that the yield of β -cortol⁵ from tetrahydro F was only 10 per cent in comparison to 6 per cent in the absence of a TPNH-generating system. It appears to us that the conclusion was unjustified either in view of the fact that in their experiments the TPNH-generating system did produce a 67 per cent increase in activity or because the low yields do not permit a decision to be made.

Direct evidence for the pyridine nucleotide dependence of the C-20-keto reductase reaction is presented in Table I. Failure of added isocitric dehydrogenase to augment a system already containing TPN, isocitrate, and manganese is attributed to endogenous isocitric dehydrogenase present in the crude enzyme. There can be little doubt, from the data presented, that the C-20 keto reduction is markedly accelerated in the presence of a TPNH-generating system.

Determination of the pyridine nucleotide specificity of the C-20-keto

⁵ See Fukushima *et al.* (6) for nomenclature

reductase reaction by direct addition of chemically reduced DPNH or TPNH was not possible because of the presence of active DPNH and TPNH oxidase activity in the microsomes. However, it was possible to maintain a continuous supply of DPNH by employing the alcohol dehydrogenase system at pH 8.8. Because of its relatively high potential, $E_0' = -0.20$ volt (18), the ethanol-acetaldehyde system cannot reduce the DPNH-DPN system, $E_0' = -0.32$ volt (19), at neutrality. However, as the pH becomes more alkaline (20) the potential of the ethanol-acetaldehyde system becomes more negative at twice the rate of that of the DPNH-DPN system. The reduction pressure of a large excess of ethanol is then able to achieve a significant reduction of DPN. The data of Table II in-

TABLE I

Dependence of Microsomal C-20-Keto Reductase on TPNH-Generating System

	C 20 ketone lost
Microsomes alone	γ
“ + TPN	None
“ + isocitrate	7
“ + TPN + isocitrate	3
“ + “ + “ + Mn^{++}	28
“ + “ + “ + “ + “ + isocitric de-	42
hydrogenase	42

Conditions: 200 eq mg of rat liver microsomes, 100 μ moles of Tris buffer, pH 7.4, 3 μ moles of $MnCl_2$, 200 μ moles of nicotinamide, 20 μ moles of isocitrate, 3 μ moles of TPN, 180 γ of cortisone, plus added isocitric dehydrogenase in the complete system. Final volume, 5.0 ml, incubation in air at 37.5° for 30 minutes.

dicates that, under conditions by which the reduction pressure (*i.e.* value of E_h) of the DPNH-DPN system was equal to that of the TPNH-TPN system, the extent of the C-20-keto reductase reaction with DPNH was only one-sixth of that with TPNH. The conclusion that the C-20-keto reductase shows a rather marked preference for TPNH appears justified.

Intracellular Localization of C-20-Keto Reductase Activity—Previous data (3) indicated that the C-20-keto reductase reaction was absent from the nuclear and mitochondrial fractions. However, it was pointed out (3) that, since the reaction appeared to require an auxiliary supply of TPNH, the failure of the nuclear and mitochondrial fractions to exhibit C-20-keto reductase activity might be due to absence of endogenous dehydrogenases for the cosubstrates used (G-6-P, isocitrate). This question was re-examined under conditions whereby TPNH was continuously supplied by the G-6-P dehydrogenase system (Fig. 1). The quantity of glucose-6-

TABLE II
Pyridine Nucleotide Specificity of C-20-Keto Reductase Reaction

Primary reductant added		Pyridine nucleotide					Potentials of pyridine nucleotide system			Cortisone lost
		Added		Present at 60 min *		Oxidized/Reduced	E_o'	E^o	E_h	
	μ -moles		μ -moles		μ -moles		volt	volt	volt	γ
Isocitrate	20	TPN	3 0	TPNH	1 3	1 7 1 3	-0 324	-0 114	-0 375	68, 66, 61
Ethanol	3440	DPN	5 5	DPNH	2 8	2 7 2 8	-0 32	-0 11	-0 374	18, 7, 10, 13, 10

The calculated reduction potential (E_h) of either pyridine nucleotide system is given by $E_h = E^o + 0.03 \log \text{oxidized/reduced} - 0.03 \text{ pH}$, in which "Oxidized" and "Reduced" are the actual experimental concentrations found for the oxidized and reduced forms of the pyridine nucleotide, pH 8.8, and the values of E^o are the corresponding E_o' values corrected to pH 1.0, according to the equation $E^o = E_o' + 0.03 \text{ pH}$. Other conditions, for both reactions, were 200 eq mg of rat liver microsomes, 180 γ of cortisone, 3 μ moles of MnCl_2 , 200 μ moles of Tris buffer, pH 8.8, 300 μ moles of nicotinamide, and 0.2 ml of ethanol. For the generation of TPNH, isocitrate, isocitric dehydrogenase, and TPN were added. For the generation of DPNH, alcohol dehydrogenase and DPN were added. Final volume, 5 ml, aerobic incubation at 37° for 60 minutes.

* Determined by measuring the extinction at 340 $\mu\mu$ of a 0.3 ml aliquot of the reaction mixture read against a similar aliquot from a blank tube containing all the constituents except the pyridine nucleotide-generating system.

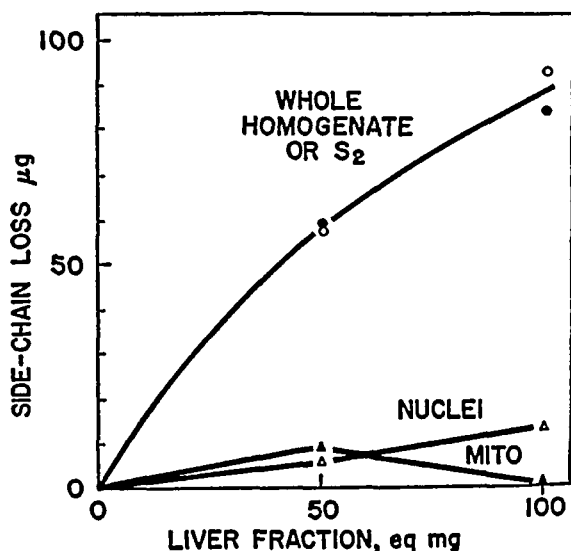


FIG 1 Recovery of C-20-keto reductase activity in S_2 fraction of rat liver. Conditions: 180 γ of cortisone, 100 μ moles of potassium phosphate buffer, pH 7.4, 20 μ moles of MgCl_2 , 15 μ moles of glucose 6-phosphate, 0.1 ml of glucose-6-phosphate dehydrogenase, 1 μ mole of TPN, 60 μ moles of nicotinamide, and 750 μ moles of sucrose, final volume, 5 ml, anaerobic incubation for 60 minutes at 37°.

phosphate dehydrogenase added was sufficient to reduce completely the added TPN in 7 minutes. The data of Fig 1 confirm that the nuclei and mitochondria are devoid of C-20-keto reductase activity. In Fig 2 are presented data obtained after prolonged high speed centrifugation of S_2 fractions in an effort to determine the distribution of the C-20-keto reductase between the microsome and supernatant fractions. There can be little doubt, in view of the data presented, that the C-20-keto reductase is in large measure associated with the microsomes. Since the protein content of the supernatant fraction is twice that of the microsomes (21),

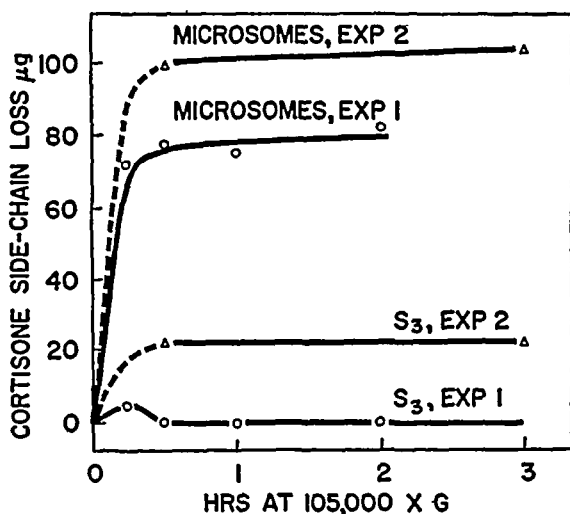


FIG 2 Recovery of C-20-keto reductase activity in the microsome fraction. Conditions: 180 γ of cortisone, 100 μ moles of Tris buffer, pH 7.4, 3 μ moles of $MnCl_2$, 20 μ moles of isocitrate, 3 μ moles of TPN, 200 μ moles of nicotinamide, final volume, 5.0 ml, aerobic incubation for 30 minutes at 37.5°. Experiments 1 and 2 refer to two separate experiments.

the enzyme is concentrated in the microsome fraction to a very high degree.

Stability of C-20-Keto Reductase and Binding to Microsomes—It was reported earlier by Glenn and Recknagel (2) and more recently by Hubener *et al* (7) that the C-20-keto reductase was unstable. Hubener *et al* attributed the loss of activity observed on fractionation of the homogenate to mechanical destruction of the microsomes. However, contrary to these reports, the C-20-keto reductase activity of rat liver microsomes has been found to be stable under a variety of experimental conditions, provided that a TPNH-generating system is added (Table III). It may be pointed out that freezing and hypotonic media are very destructive of the labile enzymes of oxidative phosphorylation associated with mitochondria. Work of Christie and Judah (22) and Dianzani (23) has indicated that rat

liver mitochondria are sensitive to CCl_4 , and work in this laboratory⁶ has also indicated that CCl_4 and other hydrocarbons in small concentration markedly alter typical biochemical properties of the mitochondria. The extreme sensitivity of mitochondria to freezing, mechanical agitation, hypotonic media, and CCl_4 and the insensitivity of the microsomal C-20-keto reductase to these treatments indicate a relatively high order of stability for this enzyme. In several experiments, C-20-keto reductase activity after lyophilization compared favorably with that of fresh microsomes. In the light of the above data pertinent to the relative stability of the C-20-keto reductase system, the earlier findings of instability can now be ascribed to loss of one or more components of an endogenous

TABLE III

Stability of Microsomal C-20-Keto Reductase

Data are given in per cent loss of activity in comparison to fresh microsomes

	Loss of C 20 keto reductase
Frozen, thawed, and analyzed immediately	No loss of activity
Washed in hypotonic medium (0.04 M nicotinamide)	" " " "
Treated with aqueous saturated CCl_4	" " " "
Stored at -10° for 4 days	29.6

Conditions: 180 γ of cortisone, 100 μ moles of potassium phosphate buffer, pH 7.4, 3 μ moles of TPN, 20 μ moles of MgCl_2 , 21 μ moles of G-6-P, 0.1 ml of G-6-P dehydrogenase (24), final volume, 5 ml, 30 minutes incubation at 38° . For the experiment with CCl_4 , the microsomes were suspended for 20 minutes at 0° in 0.25 M sucrose which had been equilibrated against CCl_4 . The microsomes were subsequently recovered by centrifugation at 2.4×10^6 g-minutes (25) and assayed.

TPNH-generating system The fact that isocitric dehydrogenase (26) and glucose-6-phosphate dehydrogenase (25) are both largely soluble enzymes supports this supposition.

In the course of a number of experiments designed to solubilize the microsomal C-20-keto reductase activity, it became evident that this enzyme is tightly bound to the microsome fraction. The enzyme could not be removed by two successive washes with the 0.25 M sucrose, 0.04 M nicotinamide medium, or with 1.0 M KCl. The enzyme resisted solubilization in a mechanical cell disintegrator. The apparatus used (courtesy of the Biochemistry Department, Western Reserve University) was essentially that described by Nossal (27) and was run for 20 seconds at 6500 cycles per minute. 8.0 ml of 25 per cent microsomes were used with 7.5 gm of ground glass. Treatment of the microsomes with ribonuclease resulted in no loss of C-20-keto reductase activity, and all the enzyme activity re-

⁶ R. O. Recknagel, unpublished

mained associated with the insoluble residue following RNAase digestion. It was found that 250 eq mg of liver microsomes required 750 γ of crystalline RNAase (Nutritional Biochemicals Corporation) for maximal digestion

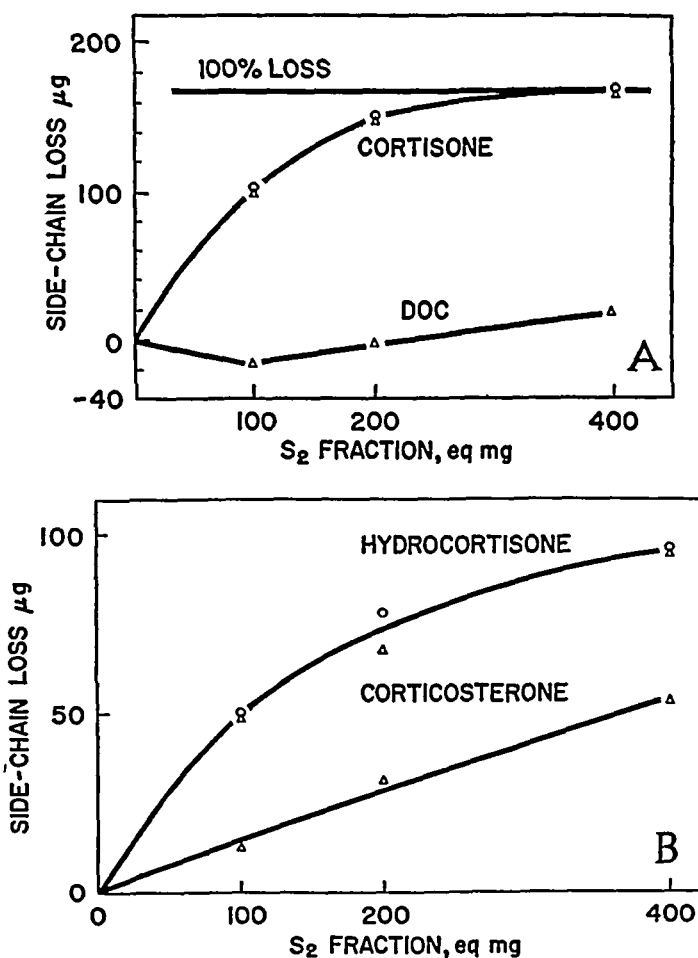


FIG 3 Substrate specificity of the C-20-keto reductase. Conditions A, same as those in Table I, except that the S_2 fraction was added as indicated. Either 180 γ of cortisone or 180 γ of DOC were added per reaction tube. Incubation at 38° for 1 hour. B, same as A, except that either 180 γ of hydrocortisone or 180 γ of corticosterone were added. O, reaction followed with phenylhydrazine (16), Δ , reaction followed with blue tetrazolium (15).

of RNA in 20 minutes at 37° (glycylglycine buffer, pH 7.0), as evidenced by the fact that no further material absorbing at E_{260} was released from the microsomes into solution with larger amounts of RNAase. It was found that C-20-keto reductase activity was destroyed when the microsomes were treated with 1 per cent, 3 per cent, or 6 per cent aqueous saponin. Aqueous 1 per cent digitonin almost completely destroyed the

enzyme, and its activity was inhibited 50 per cent at a final concentration of 0.05 per cent deoxycholate. Some success in solubilization of the enzyme has been achieved with sodium lauryl sulfate (Duponol PS, du Pont).

Substrate Specificity of C-20-Keto Reductase—Data presented in Fig. 3 indicate that the C-20-keto reductase of rat liver shows a marked preference for steroids with a characteristic 17,21-dihydroxy-20-ketone side chain and is relatively inactive against steroids lacking the 17 α -hydroxyl group.

The possibility was considered that steroids lacking the 17 α -hydroxy group would be reduced by DPNH. However, in an experiment with rat liver microsomes, it was shown that C-20 reduction of DOC proceeded to only a very small extent when DPNH was supplied via alcohol dehydrogenase at pH 8.8.

DISCUSSION

Schneider and Horstmann (28), using rat liver slices, found that side chain disappearance of various adrenocortical steroids as measured by periodate oxidation was much less marked than when measured as disappearance of Porter-Silber-reacting material. The tentative conclusion of these authors that the degradation of the side chain involved a reduction of the C-20 ketone has since been amply confirmed. Since these early experiments, two distinct but complementary lines of work on this problem have developed, one to study the intracellular localization and mechanism of the reaction in greater detail and the other to establish the nature of the reaction products. The main emphasis has been on the latter aspect of the problem. Thus, Schneider, in a subsequent study (29), presented ample evidence that the Δ^4 -3-ketone of DOC was reduced to a variety of allopregnane derivatives, but the yield of the C-20 reduction product was very low. The data of Fig. 3, A, indicating the relative inactivity of the C-20-keto reductase of rat liver toward DOC, provides an explanation at the enzyme level for this observation. The conclusion of Schneider that the α -ketol side chain is more resistant to enzymatic alteration than the Δ^4 -3-ketone must now be limited to the 17-deoxysteroids. Furthermore, since DOC is extensively reduced at C-20 by hog liver (10), the generalization must be limited to the rat. The definitive product identification studies of Caspi *et al.* (10) and of Hubener *et al.* (7) have left no doubt that hog and rat liver can extensively reduce the C-20 ketone of adrenocortical steroids. The identification of C-20-reduced steroids in human urine (6) has also established for man the importance of this reaction in the metabolism of the adrenocortical steroids.

The main line of emphasis of the work in this laboratory has been to establish the intracellular localization of the C-20-keto reductase activity.

and to establish the source of the hydrogen for the reductive step. Previous work on the question of the intracellular localization of the C-20-keto reductase has not been definitive due partly to a failure to supply reduced TPNH (3) and partly to a concentration of effort on product identification (7) rather than on an effort to obtain quantitative recovery of the enzyme activity in the various fractions equal to the unfractionated whole homogenate. Work of other laboratories (7, 10) has also not been definitive with regard to the question of the source of the hydrogen in the C-20-keto reductase reaction. No conclusions regarding the possible role of reduced pyridine nucleotides as hydrogen donors can be drawn by adding the oxidized cofactors, if an auxiliary enzyme system capable of maintaining the cofactor in the reduced state is not provided. Negative results following addition of the reduced cofactors are also not conclusive under conditions in which side reactions such as TPNH or DPNH oxidase activities may oxidatively remove the hydrogen which might otherwise serve as a source of reductive potential in the main reaction. Only under conditions in which the actual reduction potential of the pyridine nucleotide system is maintained sufficiently negative by an auxiliary reducing system and the actual concentration of the reduced cofactor is adequate for the K_m of the enzyme activating the steroid is it possible to draw any conclusions regarding either the requirement or specificity of the steroid reductase for a reduced cofactor.

SUMMARY

An adrenocortical steroid C-20-keto reductase, prepared from rat liver, will actively reduce the C-20 ketone group of cortisone in the presence of a reduced triphosphopyridine nucleotide (TPNH)-generating system. The reduction reaction exhibits a relative specificity for TPNH.

The C-20-keto reductase is predominantly associated with the microsome fraction, nuclei, mitochondria, and final supernatant fractions have little or no activity. The microsomal C-20-keto reductase activity is firmly bound to the microsome fraction.

The C-20-keto reductase exhibits a relative specificity for steroids with a 17,21-dihydroxy-20-ketone type side chain. Two 17-deoxy type steroids were relatively inactive in the C-20-keto reductase reaction.

The technical assistance of Miss Marilyn Litteria is gratefully acknowledged.

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SPECTROPHOTOMETRIC STUDIES

XVI DETERMINATION OF THE OXYGEN SATURATION OF BLOOD BY A SIMPLIFIED TECHNIQUE, APPLICABLE TO STANDARD EQUIPMENT*

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WITH A NOTE BY JULIAN B. MARSH‡

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The metabolism, as measured by oxygen consumption, of organs *in situ* is being increasingly investigated by the vascular catheterization technique (see Drabkin (3) for pertinent literature). The oxygen consumption is derived from the arteriovenous difference in the oxygen saturation or percentage of oxyhemoglobin (4) of blood entering and leaving the tissue. Such work has pointed to the need of a practical and withal reliable method for the routine analysis of the oxygen saturation of small blood samples (5, 6). Probably the most accurate determination of the percentage of oxyhemoglobin available at present is the direct spectrophotometric method of Drabkin and Schmidt (7), which avoids certain inaccuracies inherent in the standard, indirect gasometric technique employed for many years (8, 9). The extension of optical instrumentation to the analysis of oxygen saturation was made possible by the use of the Drabkin and Austin special cuvette of calibrated 0.007 cm. depth (10), which had been introduced much earlier for measurements of this type upon whole blood or concentrated hemoglobin solutions unexposed to air. An important advantage of the 0.007 cm. cuvette is that it permits the examination of undiluted blood and thereby circumvents the volumetric measurement of the sample, which remains one of the least accurate steps of analytical

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The data in this paper are largely taken from a portion of a thesis on "The optical properties of hemolyzed and whole blood," presented by Edwin Gordy to the Faculty of the Graduate School of Medicine of the University of Pennsylvania in partial fulfillment for the degree of Doctor of Medical Sciences.

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procedures upon this viscous fluid. However, the very thin layer cuvette of Drabkin and Austin, designed originally for measurements largely in the green spectral region (Fig 1, Region 2), is of delicate construction (see Drabkin and Austin (10), and Drabkin (11)) and is not ideally suited for routine practice.

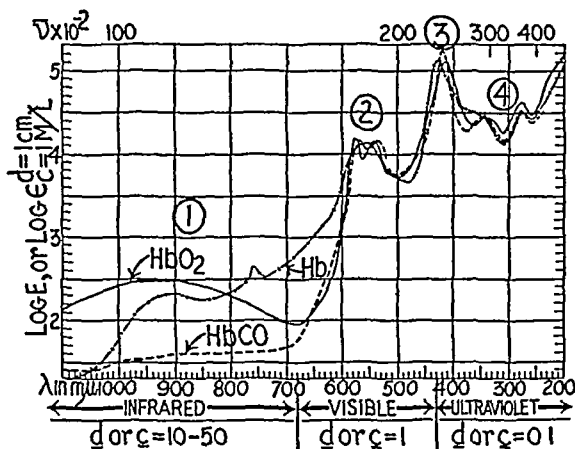


FIG 1 Absorption spectrum curves, plotted as $\log E$ (log of the molecular extinction coefficient (11)) against wave length in millimicrons, for oxyhemoglobin, HbO_2 , deoxygenated hemoglobin, Hb , and carbonyl hemoglobin, HbCO . The curves illustrate how the quantity of selective absorption in different spectral regions determines the choice of depth of layer, d , or concentration, c , to secure optimal spectrophotometric measurement of transmission, T (11). A pigment such as HbO_2 has about a 500-fold difference in density over the range of the infrared, visible, and ultraviolet regions, and three different cuvette depths with the same concentration, or three different ranges of concentration with the same cuvette depth are required for accurate measurement (11). Isosbestic points (regions of equal absorption), as at 805 and 505 $\text{m}\mu$, are evident in spectral Regions 1, 2, and 3. Each of these regions may be suitable for the determination of two or all three of these pigments when they are present together in the sample. The preferential choice of one of the regions depends upon the nature of the problem (see Drabkin (11)). Owing to the similarity of the absorption curves in Region 4, the ultraviolet region, this portion of the spectrum is obviously unsuitable for the determination of mixtures of hemoglobin derivatives.

The principles of the thin layer spectrophotometric technique in the determination of oxygen saturation (7, 10) have been the basis of a number of proposed modified methods (12-15) in which one compromise or another has been employed to attain simplification. It may be seen from the selective absorption characteristics of oxyhemoglobin, HbO_2 , and deoxygenated hemoglobin, Hb (Fig 1), that two factors, dilution and spectral region, determine the optimal depth to be used in spectrophotometry. In an appropriate spectral wave band in the red (700 to 600 $\text{m}\mu$), a cuvette of much greater depth than 0.007 cm. can be employed. In this communi-

cation a simplified direct spectrophotometric technique for the measurement of the percentage of HbO_2 in undiluted blood will be described. The procedure retains desirable features in the original Drabkin and Schmidt method (7), but is applicable to standard photoelectric photometric equipment such as the Beckman DU spectrophotometer and the recently available Bausch and Lomb Spectronic 20 instrument, with only minor modifications in the commonly employed 1 cm depth cuvettes. The reliability of this technique in comparison with the classical indirect gasometric procedure for oxygen saturation of Van Slyke and Neill (16) has been verified in extensive field trials, summarized in the "Note" appended to this paper by J B Marsh.

Equipment and Analytical Procedure

Modification of Beckman 1 Cm Cuvette—The 1 cm cuvette is converted to one with a nominal depth of 0.05 cm by means of a snugly fitting, polished, parallel glass prism (Fig 2) ¹. Our cuvette-prism combination has a calibrated optical depth of 0.067 cm, 10 times the depth of the Drabkin and Austin cuvette. An optical depth up to about 0.1 cm can be employed effectively in the technique. The modified cuvette is sealed with a stopper cut from sponge rubber (Fig 2).

Preparation of Blood-Collecting Vessel—A 6 ml capacity tonometer, as shown in Fig 2 (7), is used in the anaerobic collection and hemolysis of the blood samples. Such vessels are prepared in advance as follows: 1 ml of anticlotting hemolyzing solution is introduced into each and allowed to dry slowly on the inner surface in a thin uniform layer by gently drawing a stream of air through the tonometer. Care must be taken to avoid trapping of air bubbles during drying. The anticlotting hemolyzing solution contains 50 mg of saponin (Merck, purified), 12 mg of ammonium oxalate, 8 mg of potassium oxalate, and 2 mg of sodium carbonate per ml. The prepared tonometric vessels are connected (Fig 2, a) to a small leveling bulb which serves as a mercury reservoir, and to a male needle adapter (Fig 2, b and c). Through the reservoir, the tonometers, including their exit capillaries, are filled with thoroughly clean mercury, thereby completely expelling the air. The blood-collecting assemblies are now ready to receive the samples.

Collection of Blood Samples—The male needle adapter (Fig 2, c) is attached to a needle and, with the lower stopcock of the collecting vessel

¹ Such a prism (3 cm high, 0.98 cm wide, and 0.90 to 0.95 cm thick) can be made to specification at low cost by any good opticians' establishment, which does its own lens grinding. Only the two optical faces of the prism are polished. The snug fit of the prism in the transverse direction of the cuvette (internal width of about 0.99 cm) is essential to insure alignment of the prism and reproducibility of depth.

open and the upper stopcock closed, the needle is introduced into the blood source (vein, artery, or catheter). During ensuing operations, the mercury-leveling bulb is kept at appropriate, slight negative pressure. By means of

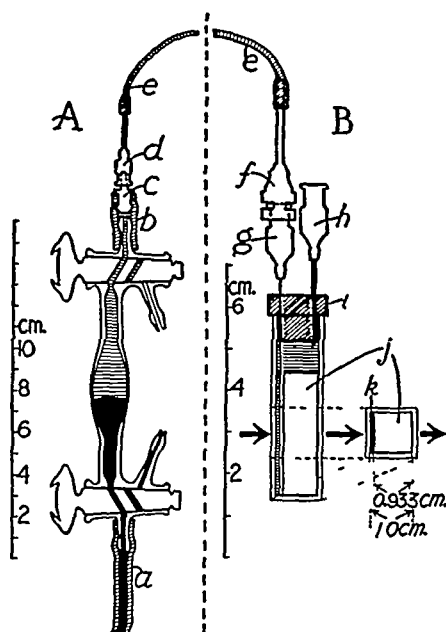


FIG 2 Sketch, drawn to scale, showing transfer of anaerobically collected and hemolyzed blood sample from glass tonometric collecting vessel of 6 ml capacity, A, into modified Beckman cuvette, B. The cuvette is represented in vertical and transverse sections, the latter with entry and exit ports (needles) removed, ready for spectrophotometric measurement. Arrows indicate the direction of luminous flux. *a*, 18 inch length of plastic tubing (Tygon, outside diameter 7 mm, inside diameter 4 mm) to mercury reservoir in small leveling bulb, held at slight positive pressure, *b*, 1 inch length of Tygon tubing (same as in *a*), connecting collecting vessel with male metal needle adapter, *c*, *d*, female half of B-D type LLX 3 inch metal needle extension (see the text), *e*, 6 inch length of thin, flexible polyethylene tubing (outside diameter 2 mm, inside diameter 1.5 mm), *f*, male half of B-D type LLX (see the text), *g*, 25 gauge, 2 inch needle (entry port), *h*, short 20 gauge needle (exit port), *i*, tightly fitting stopper cut from sponge rubber, *j*, snugly fitting polished glass slab, which reduces the optical depth of the Beckman cuvette from 1 cm to 0.067 cm, *k*, thin (0.067 cm) layer of sample.

the upper stopcock, the small amount of dead space air is eliminated through the side arm of the tonometer, which is then filled with the blood sample. The blood is allowed to displace all but 1 ml of mercury, retained to aid mixing. Both stopcocks are now closed, and the tonometer is disconnected from the blood source. The sample is thoroughly mixed and hemolyzed by gently rocking the tonometer for about 1 minute. We regard this method of preparing the blood sample as the most reliable

from the standpoint of ultimate accuracy. However, it will be evident that acceptable results can be obtained in a further simplification of technique in which the samples are collected and somewhat diluted in a syringe (see the "Note" by J B Marsh)

Transfer of Sample to Modified Cuvette—Fig 2 is practically self-explanatory as to the method of filling the cuvette. The connection between the collecting vessel, *A*, and the cuvette, *B*, is accomplished by means of the connector, *d*, *e*, and *f*, and a 2 inch, 25 gauge needle, *g*. The connector is made by cutting a 3 inch B-D type LLX metal needle extension into equal length halves (female *d*, and male *f*), filing the cut ends smooth, and joining them with a 6 inch length of thin flexible polyethylene tubing, *e* (outside diameter 2 mm, inside diameter 1.5 mm). The male end of the connector is attached to the needle, *g*, which is inserted through the sponge rubber stopper, *i*. The point of this needle should touch the bottom of the cuvette. A short 20 gauge needle, *h*, inserted through the stopper (Fig 2), is a vent for the escape of air and excess blood, as the cuvette is slowly and completely filled from the bottom up with the sample. During the transfer of the sample the tonometer is held vertical at slight positive pressure. About 1 ml of blood is needed for filling the modified cuvette, since the tonometer contains 5 ml, the sample is sufficiently large to allow for a liberal overflow through *h*. This provision insures samples uncontaminated by air. After the cuvette has been properly filled, both needles are removed and the sample is ready for measurement.

Spectrophotometry—Measurements are carried out in the usual manner with the Beckman DU spectrophotometer. The method depends upon optical density (*D*) readings at two wave lengths, 660 and 805 $m\mu$. The narrowest possible slit is used, namely 0.015 to 0.02 mm, which corresponds to a spectral band width (span or wave band) of 2.5 to 3 $m\mu$. Fig 1, showing the absorption spectrum curves of HbO_2 , Hb, and HbCO in the infrared, visible, and ultraviolet regions, supplies the information upon which the choice of spectral region and cuvette depth is based (11). It may be seen that wave length 660 $m\mu$ is a region of large spectroscopic difference between HbO_2 and Hb, whereas at 805 $m\mu$ the absorption of the two pigments is isosbestic (11), i.e. the same. The measurement in the latter region is an effective and convenient substitute for an independent determination of total pigment, but is utilized with the assumption that one is dealing only with mixtures of the two pigment species, HbO_2 and Hb. This assumption of a two-component system is inherent in the proposed method of handling the data (below) and appears to hold for most blood samples, which normally contain only traces of methemoglobin, MHb (4, 7) and, with the possible exception of samples from heavy smokers (9), only negligible amounts of carbonyl hemoglobin, HbCO.

The spectrophotometric constants, ϵ values, supplied in Tables I to III, are *fractional molar extinction coefficients* (11)²

Determination of Percentage of HbO₂—A knowledge of the optical density, D , of the sample at wave lengths 660 and 805 $m\mu$ and the spectrophotometric constants of HbO₂ and Hb in these spectral regions, obtained from Table I, are all that is required to calculate the per cent of HbO₂ present. The validity of the use of these constants and the working equation (Equation 8, below) based upon them may be inferred from the good agreement between results obtained by this method and by the gasometric procedure. However, the four groups of investigators who participated in the trial of the optical method used the Beckman DU spectrophotometer (see the "Note" by J B Marsh). It should be cautioned that the ϵ values are not necessarily transferable to photometric equipment or conditions of measurement which do not allow the narrow spectral isolation used by us. For those employing filter photometers or wide spectral bands for measurement, Equation 5 (below) may be used by substituting in it the values for ϵ determined in the particular instrument. The development of the working equation parallels that of Drabkin (11) for mixtures of carbonyl and oxyhemoglobin and is applicable to any two-component mixture at any two appropriate wave lengths, with at least one of which advantage may be taken of a large difference in the spectral absorption between the two components. The following symbols are used: A and B , the two components, $\epsilon_{\lambda 1A}$, $\epsilon_{\lambda 2A}$, $\epsilon_{\lambda 1B}$, and $\epsilon_{\lambda 2B}$, the extinction at definite concentration and depth of layer for the respective components, established at the two wave lengths, λ_1 and λ_2 , x , the fraction of component A , and $1 - x$, the fraction of component B , r , the ratio of optical densities, $D_{\lambda 1}$ and $D_{\lambda 2}$, obtained by measurement on the mixture. The relationship between r , x , and extinction coefficients may be at once formulated as

$$r = \frac{x\epsilon_{\lambda 1A} + (1 - x)\epsilon_{\lambda 1B}}{x\epsilon_{\lambda 2A} + (1 - x)\epsilon_{\lambda 2B}} \quad (1)$$

Solving for x , dividing by $\epsilon_{\lambda 2B}$ and collecting terms, the yield is

$$x = \frac{r - \frac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}}{r \left(1 - \frac{\epsilon_{\lambda 2A}}{\epsilon_{\lambda 2B}} \right) + \left(\frac{\epsilon_{\lambda 1A}}{\epsilon_{\lambda 2B}} - \frac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}} \right)} \quad (2)$$

Equation 2 applies to the general case, but in this particular instance $\epsilon_{\lambda 2A}$

² ϵ , at a concentration of 1 mmole per liter, referable to a 1 iron atom, equivalent weight of 16,700 for hemoglobin, and a cuvette depth of 1 cm. The concentration in all cases was determined spectrophotometrically upon aliquots converted into cyanmethemoglobin with the constant $\epsilon = 11.5$ at wave length 540 $m\mu$ (17).

and $\epsilon_{\lambda 2B}$ are identical (isosbestic absorption at 805 $m\mu$ (Table I)) and the first term in the denominator drops out, yielding

$$x = \frac{r - \frac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}}{\frac{\epsilon_{\lambda 1A} - \epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}} \quad (3)$$

For practical convenience in later handling in this particular instance, the signs are reversed, obtaining

$$x = \frac{\frac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}} - r}{\frac{\epsilon_{\lambda 1B} - \epsilon_{\lambda 1A}}{\epsilon_{\lambda 2B}}} \quad (4)$$

Equation 4 is now rewritten specifically to obtain Equation 5, applicable to the photometric determination of the *fraction of HbO₂* by any optical equipment, when one of the two wave lengths used for measurement is an isosbestic region for the two components

$$\text{Fraction of HbO}_2 = \frac{\frac{\epsilon_{660}\text{Hb}}{\epsilon_{805}\text{Hb}} - \frac{D_{660}}{D_{805}}}{\frac{\epsilon_{660}\text{Hb} - \epsilon_{660}\text{HbO}_2}{\epsilon_{805}\text{Hb}}} \quad (5)$$

The established ϵ values at wave lengths 660 and 805 $m\mu$ (Table I) are substituted in Equation 5 to give

$$\text{Fraction of HbO}_2 = \frac{\frac{0.820}{0.196} - \frac{D_{660}}{D_{805}}}{\frac{0.820 - 0.100}{0.196}} \quad (6)$$

which becomes

$$\text{Fraction of HbO}_2 = \frac{4.18 - \frac{D_{660}}{D_{805}}}{3.67} \quad (7)$$

Therefore,

$$\% \text{ HbO}_2 = \left(\frac{4.18 - \frac{D_{660}}{D_{805}}}{3.67} \right) 100 \quad (8)$$

It is obvious that the *per cent of Hb* in the mixture is obtained from 100 less *per cent of HbO₂*, as given by the working Equation 8. It should be

evident that the exact optical depth of the cuvette employed need not be known for the determination of per cent of HbO_2 by the present method, provided that a reliably calibrated spectrophotometer affording narrow spectral isolation, such as the Beckman DU instrument, is employed, that the cuvette depth is between 0.05 and 0.10 cm, permitting the instrument to be used over its most accurate density range, when measuring undiluted samples of blood, and that the spectrophotometric constants established by us (Table I) are accepted as applicable. On the other hand, for the establishment of the ϵ values and for the determination of total pigment as cyanmethemoglobin, an accurate calibration of cuvette depth is necessary.

EXPERIMENTAL

The ϵ values in Table I were derived from measurements with the Beckman DU spectrophotometer and our modified 0.067 cm depth cuvette on nine samples of fresh human blood. The fully oxygenated specimens (HbO_2) were obtained by equilibrating 10 ml volumes of the hemolyzed blood in an atmosphere of oxygen in a 400 ml capacity tonometer. The deoxygenated samples (Hb) were obtained by adding sufficient active dithionite, $\text{Na}_2\text{S}_2\text{O}_4$ (Eimer and Amend, low iron content), in solid form.

In published simplifications (12-14) of the original Drabkin and Schmidt technique (7), the possibility has been neglected that under certain conditions blood samples may contain more than minimal quantities of carbonyl hemoglobin, HbCO , or methemoglobin, MHb . In such cases the above method, designed for a two-component mixture of HbO_2 and Hb, would yield erroneous information. With a view of extending the technique to the determination of the per cent of HbO_2 in the presence of HbCO or MHb , the extinction coefficients for HbCO , MHb_{acid} (pH 6.1), and $\text{MHb}_{\text{alkaline}}$ (pH 8.8) were obtained for the red and near infrared spectral regions, wave lengths 620 to 900 $\text{m}\mu$ (Table II). The data in Table II disclose that wave length 805 $\text{m}\mu$ can no longer serve for the total pigment estimation in hemolyzed blood samples containing several per cent of HbCO or MHb . At 805 $\text{m}\mu$ the extinction of radiant flux by HbCO is only one-fifth that of HbO_2 or Hb, whereas, dependent on the pH of the sample, MHb in this spectral region will have more than 2-fold greater absorption than either the HbO_2 or Hb species. However, it may also be seen (Tables I and II) that the spectral absorptions of HbO_2 and HbCO are practically identical at wave length 660 $\text{m}\mu$. Moreover, at this wave length and at an intermediate pH of 8.0, the extinction coefficients of Hb and MHb are virtually the same (Table I and Table II, footnotes). This information is utilized in the following further modification, designed by one of us (D. L.

D), to afford effective correction for the presence of HbCO or MHb in blood suspected of containing abnormal amounts of either of these derivatives. It is assumed that ϵ values at the wave length of 805 $m\mu$ less than 0.196 will reflect the presence of HbCO, whereas values greater than 0.196

TABLE I

Fractional Molar Extinction Coefficients, ϵ , in Red and Near Infrared Spectral Regions for HbO₂ and Hb Obtained from Nine Samples of Whole Hemolyzed Blood*

HbO₂, sample oxygenated, Hb, sample treated with Na₂S₂O₄, measurements in modified cuvette of 0.067 cm depth with Beckman DU spectrophotometer, spectral span = 2.5 to 3 $m\mu$

Wave length <i>mμ</i>	HbO ₂		Hb		Wave length <i>mμ</i>	HbO ₂		Hb	
	ϵ	S D †	ϵ	S D †		ϵ	S D †	ϵ	S D †
600	1.06	0.03 ±0.01	3.40	0.03 ±0.01	755	0.129	0.011 ±0.003		
605	0.674	0.013 ±0.003	2.61	0.05 ±0.01	760			0.378	0.006 ±0.002
610	0.464	0.011 ±0.003	1.96	0.04 ±0.01	800	0.189	0.010 ±0.003	0.200	0.007 ±0.002
625	0.228	0.011 ±0.003	1.22	0.04 ±0.01	805	0.196‡		0.196‡	
650	0.118	0.006 ±0.002	0.872	0.019 ±0.005	810	0.202	0.010 ±0.003	0.191	0.005 ±0.001
660	0.100	0.018 ±0.004	0.820	0.006 ±0.002	850	0.233	0.005 ±0.001	0.167	0.005 ±0.001
695	0.089	0.014 ±0.003	0.475	0.016 ±0.004	900	0.256	0.006 ±0.002	0.170	0.003 ±0.001
735	0.104	0.004 ±0.001	0.307	0.009 ±0.002					

* See the text, footnote 2

† Standard deviation = $\sqrt{\sum d^2/(n-1)}$, the values after plus-minus are the standard errors of the standard deviation = S D / $\sqrt{2n}$

‡ Obtained by interpolation

will indicate the presence of MHb (Tables I and II). Since such ϵ values are obtained from D_{805}/cd , where c is the concentration (in millimoles per liter) and d the cuvette depth in cm, the modified procedure demands that the concentration of total hemoglobin pigments be determined independently on an aliquot of the sample converted into cyanmethemoglobin and that a cuvette of exactly calibrated depth be used. Another aliquot, or the remainder of the sample, unexposed to air, is hemolyzed and at the same time adjusted to pH 8.0 by including appropriate buffer solution with

the saponin in the preparation of the tonometers. In the handling of the data three quantities are needed, $D_{660, \text{pH } 8.0}$, $D_{805, \text{pH } 8.0}$, and theoretical $D_{805, \text{pH } 8.0}$ (for a mixture of HbO_2 and Hb). The first two are obtained by measurement, and the last is derived from $D_{805, \text{pH } 8.0} = \epsilon_{805, \text{Hb}} c d = 0.196 c d$. The ratio, $D_{660, \text{pH } 8.0}$ (measured) to $D_{805, \text{pH } 8.0}$ (derived), is substituted in

TABLE II

Fractional Molar Extinction Coefficients, ϵ , in Red and Near Infrared Spectral Regions for HbCO, Acid MHb, and Alkaline MHb†*

Measurements in unmodified Beckman cuvette 1 cm in depth on solutions prepared from hemolyzed washed red cells at a concentration of 0.680 mmole per liter, with a spectral span of 2.5 to 3 μ , HbCO samples obtained by equilibration with alkali- and water-washed illuminating gas, MHb samples prepared by addition of $\text{K}_3\text{Fe}(\text{CN})_6$ to appropriate buffered solutions at 0.1 ionic strength (see Austin and Drabkin (18))

Wave length	HbCO	MHb _{acid} †	MHb _{alkaline} ‡	Wave length	HbCO	MHb _{acid} †	MHb _{alkaline} ‡
$m\mu$	ϵ	ϵ	ϵ	$m\mu$	ϵ	ϵ	ϵ
620	0.424	3.38	3.24	760	0.041	0.198	0.360
630		3.88	2.14	800	0.041	0.326	0.488
640	0.207	3.85	1.53	805	0.040	0.345	0.506
650		2.45	1.24	810	0.040	0.364	0.523
660	0.105	1.05	0.517	820	0.040	0.403	0.544
675		0.320	0.320	840	0.040	0.475	0.517
680	0.068	0.224	0.301	845		0.499	0.502
720	0.047	0.131	0.336	880	0.040	0.601	0.351
740		0.155	0.345	900	0.040	0.660	0.298

* See the text, footnote 2

† Data obtained by one of us (D. L. D.)

‡ pH 6.1, measured by glass electrode

§ pH 8.8, measured by glass electrode. ϵ for MHb at intermediate pH between 6.1 and 8.8 may be calculated (18), ϵ for MHb at pH 8.0 = 0.826 at $\lambda 660 m\mu$ and 0.412 at $\lambda 805 m\mu$

Equation 8, which is now used to calculate the per cent of HbO_2 . This relatively simple procedure for obtaining the per cent of HbO_2 in a mixture of HbO_2 , Hb, and MHb is possible because the absorption spectra of HbO_2 and Hb are invariant with pH and only that of MHb is pH-dependent (18), and because the adjustment of the pH to 8.0 renders identical the extinction coefficients of Hb and MHb. Owing to the near identity of the ϵ values (at 660 $m\mu$ and at pH 8.0) of Hb and MHb on the one hand, and of HbO_2 and HbCO on the other, the evaluation of Hb in a mixture of HbO_2 , Hb,

and MHb or of HbO₂ in a mixture of HbO₂, Hb, and HbCO requires the use of appropriate correction factors, supplied in the following formulations

$$\% \text{ MHb} = \left(\frac{\epsilon_{805, \text{pH } 8.0} - 0.196}{0.216} \right) 100 \quad (9)$$

$$\% \text{ HbCO} = \left(\frac{\epsilon_{805, \text{pH } 8.0} - 0.196}{0.156} \right) 100 \quad (10)$$

$\epsilon_{805, \text{pH } 8.0}$ is obtained from the measurement of $D_{805, \text{pH } 8.0}$, since $\epsilon_{805, \text{pH } 8.0} = D_{805, \text{pH } 8.0}/cd$. In the above equations the numerators represent, respectively, the partial change in extinction between HbO₂ and MHb_{pH 8.0} and between HbO₂ and HbCO, whereas the denominators are, respectively, the total change or difference in ϵ between the components ($\epsilon_{\text{MHb, pH } 8.0} - \epsilon_{\text{HbO}_2} = 0.412 - 0.196$ and $\epsilon_{\text{HbO}_2} - \epsilon_{\text{HbCO}} = 0.196 - 0.04$). The method is not applicable to the simultaneous presence of both HbCO and MHb, but this situation should be encountered only rarely. The absorption curves in Fig. 1 suggest that wave length 1050 m μ should be an ideal region for the determination of HbO₂ in mixtures of HbO₂, Hb, and HbCO, since the two latter species are isosbestic at this wave length. However, the Beckman DU spectrophotometer and other usually available equipment are inaccurate beyond 900 to 1000 m μ (11).

Table III furnishes extinction coefficient values for HbO₂ and Hb, obtained with the Bausch and Lomb Spectronic 20 spectrophotometer, used in combination with the Arthur H. Thomas Roto-Cell assembly. For the present purpose the latter adjunct is essential. It is a water-cooled carrier which permits both the use of parallel side cuvettes of exact optical depth and the rapid interchange in the light path of blank and sample.³ While the ϵ values yielded by this equipment are not identical with those obtained with the Beckman DU spectrophotometer, nevertheless, as plot-

³ The wave length scale of the Spectronic 20 instrument should be calibrated by the user and, if necessary, reset. An ϵ value of 11.5 at 540 m μ for cyanmethemoglobin was found to be applicable to the Spectronic 20 instrument, provided with the blue-sensitive phototube, R. M. A. type 5581, and the Thomas double chambered cuvette of Corex brand glass. Solutions of cyanmethemoglobin of known concentration were then used for the calibration of the depth of modified cuvettes. For modifying the depth of each chamber of the cuvette, a pair of very snugly fitting glass prism spacers, 0.93 cm. in optical depth and 2.8 cm. high, was ground and polished to our specifications by the Arthur H. Thomas Company. The calibrated optical depth of the modified partitioned cuvette was 0.0697 cm. With these particular spacers, 0.3 ml. of hemolyzed blood will not only fill the optical area but will allow for 0.2 ml. of sample above the spacers. It is possible to use spacers of the same height as the cuvette, and under these conditions about 0.12 ml. of blood will suffice.

ting of the data in Tables I and III will show, the relatively inexpensive Spectronic 20 spectrophotometer does a good over-all job in furnishing the absorption patterns of HbO_2 and Hb. Hence, the equipment can serve for a relatively accurate determination of per cent of HbO_2 in hemolyzed blood samples unexposed to air. It may be calculated that the ratio of $\epsilon_{\text{Hb}}/\epsilon_{\text{HbO}_2}$ is very slightly greater here (Table III) at 650 than at 660 $m\mu$, and the isosbestic point is at 810 rather than at 805 $m\mu$. The ϵ val-

TABLE III

Fractional Molar Extinction Coefficients, ϵ , in Red and Near Infrared Spectral Regions for HbO_2 and Hb Obtained on Whole Hemolyzed Blood†*

HbO_2 , sample oxygenated, Hb, sample treated with $\text{Na}_2\text{S}_2\text{O}_4$, measurements with the Bausch and Lomb Spectronic 20 diffraction-grating spectrophotometer, provided with red and infrared sensitive phototube, type IP40, a Corning red glass for filtering out interference from the second order spectrum produced by the grating, Arthur H. Thomas Roto-Cell assembly, which accommodates the partitioned cuvette, here modified to a calibrated depth of 0.0697 cm. by means of glass prism spacers and a voltage stabilizer.

Wave length <i>mμ</i>	HbO_2	Hb	Wave length <i>mμ</i>	HbO_2	Hb
	ϵ	ϵ		ϵ	ϵ
600	0.848	2.74	750	0.183	0.459
610	0.530	1.96	760	0.198	0.454
620	0.348	1.50	800	0.248	0.285
630	0.263	1.24	805	0.254	0.269
650	0.178	0.997	810	0.261	0.263
660	0.165	0.913	850	0.304	0.254
700	0.143	0.537	900	0.337	0.267
740	0.176	0.417			

* See the text, footnote 2

† Data obtained by one of us (D. L. D.)

ues at wave lengths 650 and 810 $m\mu$ are used as in Equation 5, and the following working equation applicable to the Spectronic 20 instrument is derived

$$\% \text{HbO}_2 = \left(\frac{3.79 - \frac{D_{650}}{D_{810}}}{3.11} \right) 100 \quad (11)$$

Because of the comparatively small volumetric size of the optical compartments of the Thomas cuvette, this equipment is particularly suitable for small blood samples.

*Note on Results with Present Optical Method in Comparison with
Those by Gasometric Technique*

BY JULIAN B MARSH

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The technique of Gordy and Drabkin was adapted for use with ordinary syringes. The method of obtaining and hemolyzing the blood was essen-

TABLE IV
*Comparison of Results by Gordy and Drabkin Spectrophotometric Procedure
with Those by Gasometric Technique*

Investigators	No of individual sample comparisons	Mean difference in per cent saturation between spectro photometric and gasometric analysis	Standard error of the mean difference
		<i>per cent</i>	
Marsh, J B , Khouri, E , and Jetton, M *	39	+0 62	±0 579
Williams, M H *	86	+1 97	±0 368
Cooper, D Y , Billman, D E , and Cooper, H R †	29	+1 00	±0 593
Wyeth, J , Ecker, P , and Polis, B D ‡	14	+0 41§	±0 252

* From the Department of Cardiorespiratory Diseases, Army Medical Research Institute, Washington, D C

† From the United States Naval Medical School, Bethesda, Maryland

‡ From the Aviation Medical Acceleration Laboratory, United States Naval Air Development Center, Johnsville, Pennsylvania

§ Calculated from the data of Wyeth, Ecker, and Polis (19), assuming an oxygen capacity of 20 volumes per cent for all samples

tially that of Hickam and Frayser (14), except that the saponin was introduced into the syringe through a 3-way metal stopcock. After hemolysis, a 3 inch, 22 gauge needle, bent at right angles, was attached, and the sample was delivered at the bottom of a Beckman cuvette 1 cm deep but modified to a depth of 0.10 cm by means of a lucite (Plexiglas) block, 9 × 9 mm in cross section and 3.5 cm high. It was convenient to place a small screw in the top of the lucite block so that it could be removed easily. Such plastic prisms were inexpensive substitutes for the polished glass prisms used by Gordy and Drabkin, and were discarded when their optical faces became marred after a period of service.

Measurements of the optical density at 660 and 805 mμ were made with

the Beckman DU spectrophotometer, and the per cent saturation (per cent of HbO_2 of the total hemoglobin) was calculated by means of the working Equation 8, based on the constants established by Gordy and Drabkin (Table I). The optical density readings remained unchanged for at least 15 minutes, and presumably it was not necessary to prevent exposure of the solution above the light path to the air, as in the cuvette described by Nahas (13). The chief source of difficulty in the present method was in the unreliability of commercially available saponin preparations. We found some samples of Merck and Eastman Kodak saponin to be satisfactory. With hemolytically potent saponin preparations, it was not necessary to use a 30 per cent solution, the concentration employed by Hickam and Frayser (14). In many of the determinations (Table IV), the blood was hemolyzed with 0.2 volume of 10 per cent saponin (Eastman Kodak, special) buffered at pH 7.4 with phosphate buffer. Wyeth, Ecker, and Polis (19) used a detergent, Triton X 100 (0.05 volume of a 33 per cent solution in 0.1 M borax), as the hemolytic agent.

The data in Table IV are a summary of results in four independent field trials of the Gordy and Drabkin method, carried out during the past 4 years. In confirmation of earlier findings (9), the per cent of oxygen saturation was slightly higher by the spectrophotometric than by the classical Van Slyke and Neill (16) gasometric method. However, the agreement of the independent techniques was most satisfactory.

DISCUSSION

The present simplified spectrophotometric method for the determination of the per cent of HbO_2 in hemolyzed blood unexposed to air retains the essential desirable features in the original Drabkin and Schmidt technique (7). The method is easily adaptable to standard equipment, such as the Beckman DU spectrophotometer and the new inexpensive Bausch and Lomb Spectronic 20 instrument. The manipulative steps involved are simple and rapid, and only small blood samples are required. Disadvantages in other modifications of the Drabkin and Schmidt thin layer technique (13, 14) have been overcome.

Under optimal conditions, a single component of a mixture of several species, the absorption constants of each of which have been established, can be determined spectrophotometrically with an accuracy of 1 per cent (7, 11, 18). The agreement between per cent of oxygen saturation (i.e. per cent of HbO_2 of total hemoglobin) determined independently by the present, direct spectrophotometric technique and that determined by the indirect gasometric method was easily within 2 per cent.

SUMMARY

A simple, rapid, and accurate method for the spectrophotometric analysis of the per cent of HbO_2 in hemolyzed blood unexposed to air has been described

The method is applicable to standard equipment, such as the Beckman DU and the Bausch and Lomb Spectronic 20 spectrophotometers

Suitable modifications in procedure permit the per cent of HbO_2 to be determined also in blood samples which may contain more than negligible amounts of carbonyl or methemoglobin

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STUDIES ON THE ENZYME ENOLASE*

I EQUILIBRIUM STUDIES

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Enolase catalyzes the interconversion of D-glyceric acid 2-phosphate and enolpyruvic acid phosphate,¹ and, as a participant of Embden-Meyerhof glycolysis and fermentation, is widely distributed in living cells. The enzyme was first purified by Warburg and Christian (1), who demonstrated its metal activation and made the initial studies of the kinetics and the thermodynamics of the enolase reaction. More recently, Malmstrom (2-6) has extended that work, with special emphasis on the effect of the activating metals. Previous studies on enolase have employed synthetic DL-glyceric acid 2-phosphate as the substrate, and the interpretation of results has been complicated by this fact. A recent unequivocal synthesis leading to pure D-GA2P (7) makes available both substrates of enolase as the pure natural isomers.

The enolase system is particularly useful for the study of the mechanism of metal activation. The equilibrium is near enough to 1 so that both the forward and the reverse reaction can be studied, the enzyme is activated by at least seven different metals, and the interconversion of the substrates may be followed spectrophotometrically.

This paper describes in detail the factors affecting the ultraviolet absorption characteristics of enolpyruvic acid phosphate so that the assay method can be made more precise. The binding constants for complexes between the substrates and several metal ions were determined, and the apparent equilibrium constants at different metal and hydrogen ion concentrations were related to the metal binding and the pK values of the ionizable groups in the substrates.

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¹ The following abbreviations are used: GA2P, glyceric acid 2-phosphate, GA3P, glyceric acid 3-phosphate, PAP, enolpyruvic acid phosphate, TPAI, tetra-*n*-propylammonium iodide.

EXPERIMENTAL

Materials—The trisodium salt of D-GA2P was synthesized by the method of Ballou and Fischer (7), and PAP was prepared according to the procedure of Baer and Fischer (8). The resulting water-insoluble and light-sensitive silver barium salt was converted to the new, stable, water-soluble cyclohexylammonium salt, as described below. Enolase was prepared as the crystalline mercury salt according to the method of Warburg and Christian (1), starting with brewers' yeast (generously supplied by the Goebel Brewing Company, Oakland, California). The activity of the purified enzyme was comparable with the values in the literature. The dialyzed mercury-free solution was stored in small samples (0.05 to 0.1 ml) at -4° . When used, each sample was diluted with distilled water and the dilute solution was kept on ice during the experiment and stored in the refrigerator. When treated in this way, the activity of the dilute samples remained undiminished for several days.

All the other chemicals used were either commercial samples of highest purity or synthetic compounds, recrystallized before use.

Preparation of Cyclohexylammonium Enolpyruvic Acid Phosphate—1.9 gm of a twice crystallized sample of silver barium enolpyruvic acid phosphate (8) in a 40 ml centrifuge tube were suspended in 15 ml of water, and 1.0 N hydrochloric acid (4.36 ml), equivalent to the silver ion, was added. The mixture was stirred for 5 minutes, then the silver chloride was centrifuged and the supernatant fluid decanted into a clean 40 ml centrifuge tube. The silver chloride was washed with 5 ml of water, and this water was combined with the decanted solution. The barium ions in the solution were then precipitated by the addition of equivalent 1.0 N sulfuric acid (8.75 ml) and the mixture was again centrifuged. The supernatant fluid was filtered, if necessary, by suction through a Whatman No. 50 paper to remove floating particles of barium sulfate, the filtrate then was brought to pH 8 with cyclohexylamine. This solution was concentrated to dryness *in vacuo* at a bath temperature of 40° , giving a white crystalline residue. The crude cyclohexylammonium salt was redissolved in 15 ml of warm water (60°), and the solution was diluted with acetone to turbidity. After 3 hours at 5° , more acetone was added to complete crystallization, and the mixture was left overnight at 5° . The crystalline product was collected on a Buchner funnel and washed with acetone. The crystals were dried in air, and then in a vacuum desiccator over phosphorus pentoxide for 1 day. The yield was 1.35 gm. The mother liquor was concentrated to dryness and the residue was redissolved in 5 ml of warm water and diluted with acetone. After 20 hours at 5° , a second crop of 0.5 gm was obtained. The total yield was 1.85 gm.

(91 per cent) A sample, recrystallized and dried as above, slowly decomposed between 155–180°

$C_{21}H_{44}O_6PN_3$ (465)	Calculated	N 9.01, P 6.65
	Found	" 9.24, " 6.48

The biological purity of this compound has been tested by measuring the oxidation of reduced diphosphopyridine nucleotide in the presence of adenosine diphosphate, pyruvic phosphokinase, and lactic dehydrogenase. 1 mole of the enolpyruvic acid phosphate resulted in the oxidation of 0.97 mole of reduced nucleotide. Therefore, it is felt that the silver barium salt (Baer procedure) and the cyclohexylammonium salt are of equal purity, and that the ultraviolet absorption characteristics recorded in this paper (Table III, and Figs. 1, 2, and 3) more nearly approximate those for the pure compound than the values recorded previously (1). The cyclohexylamine salt is preferred because of its solubility and stability and may be used as such in most enzymatic studies, although removal of the amine can be easily accomplished by treatment with the appropriate ion exchange resin.

Apparatus and Methods—The pH of all solutions was determined with a Beckman pH meter or a Leeds and Northrup pH indicator, both with the usual calomel and glass electrodes.

The titration experiments were carried out with the automatic recording apparatus of Neilands and Cannon (9). The gas phase was N_2 and the temperature 25°. The compounds to be titrated were converted into the free acids by treatment with Dowex 50 (H^+) and titrated at a concentration of 1 μ mole per ml in a solution containing tetra-*n*-propylammonium iodide to give constant ionic strength, and with 0.5 *N* tetra-*n*-propylammonium hydroxide as the base. The pK_a' values were read directly from the titration curves. The use of the tetraalkylammonium salt and base has been recommended by Smith and Alberty (10) for the determination of binding constants from titration data. They showed that the alkali metals will bind phosphate esters to a considerable extent and thus complicate the study of the binding of other metals, whereas the larger tetraalkylammonium ions do not form complexes significantly with the esters.

The binding constants were estimated according to the method of Smith and Alberty (10, 11), who showed that at constant ionic strength the shift in the apparent dissociation constant of an acid, caused by the addition of metal, is related to the metal concentration and the binding constant for the acid-metal complex in the following way

$$pK_a' \text{ (no metal)} - pK_a' \text{ (metal)} = \log (1 + D [M]) \quad (1)$$

where D designates the binding constant or stability constant ($D = [AM]/[A][M]$), $[A]$ the acid concentration, and $[M]$ the metal concentration

The determinations of the apparent equilibrium constant were based on the ultraviolet absorption of PAP (1). Most of the equilibrium determinations were performed starting with PAP. The optical density at start and at equilibrium was determined with a Beckman DU spectrophotometer, and the apparent equilibrium constant was obtained directly from these values after proper correction for the absorption of the enzyme

TABLE I

Apparent Dissociation Constants of GA2P and PAP Determined at Two Ionic Strengths

The data are not corrected for the water blank

		Ionic strength 0.1	Ionic strength 0.4
GA2P	pK_2'	3.55	3.6
	pK_3'	7.0	7.1
PAP	pK_2'	3.4	3.5
	pK_3'	6.35	6.4

and buffer. When the equilibrium was approached from GA2P, the extinction coefficient for PAP for each set of conditions was determined.

RESULTS AND DISCUSSION

Titration of GA2P and PAP—The apparent dissociation constants of GA2P and PAP at ionic strength 0.1 and 0.4 are shown in Table I. Since the method is not reliable below pH 2, the dissociation of the primary phosphate group is not included. The free acids were titrated in 5 ml aliquots of a 1 mM solution, in a medium containing tetra-*n*-propylammonium iodide to constant ionic strength. The binding constants were determined by substituting metal for TPAI to give the same ionic strength, and then determining the shift in the pK_a' values caused by the addition of the metal. The binding constants determined from this shift in pK_a' according to Equation 1 are given in Table II. Equation 1 is derived on the assumption that only the totally ionized species of the substrates bind metals. This assumption seems valid in the present case, since the addition of metals caused no significant change in the second dissociation of the substrates. The binding constants did not vary significantly over the ionic strength range from 0.1 to 0.4. The data in Table II show that the assumption made in the past, namely that the metal binding to the two substrates is of equal magnitude (4), is incorrect.

Ultraviolet Absorption of Analytically Pure PAP—The results from our initial measurements of the extinction coefficient of PAP are compared with those of Warburg and Christian in Table III. The more detailed study of the effect of pH and magnesium ion concentration on the extinction coefficient is summarized in Figs. 1 to 3.

In Fig. 1 is given the variation of the extinction coefficient in the physio-

TABLE II

Binding Constants for Complexes of GA2P and PAP with Several Metals
Determined from titration data according to Equation 1

	$D_{GA2P'}$ (1 per mole)	$D_{PAP'}$ (liters per mole)
Potassium	15	12
Magnesium	280	180
Manganese	1225	560
Zinc	2500	920
Cadmium	2500	920
Cobalt	920	350
Nickel	760	220

TABLE III

Extinction Coefficients ($E \times 10^{-3}$ Liters per Mole per Cm) of Enolpyruvic Acid Phosphate in 0.05 M Phosphate Buffer

Wave length, mμ	Present investigation			From Warburg and Christian (1)		
	pH 6	pH 7	pH 8	pH 6.4	pH 7.4	pH 8.1
220	3.43	3.74	3.78			
230	2.18	2.91	3.02		3.0	
240	0.94	1.44	1.54	1.41	1.73	1.80
250	0.24	0.33	0.36		0.43	
260	0.06	0.06	0.06		0.10	

logical pH range corresponding to the change from $PAPH^{2-}$ to PAP^{3-} . Clearly, the extinction coefficient of the protonated form is less than that of the totally ionized form, and, at any pH at which both species are present, the observed extinction coefficient is determined by

$$E_{\text{observed}} = \frac{E_1 + ([H^+]/K_a')E_2}{1 + [H^+]/K_a'} \quad (2)$$

where E_1 and E_2 are the extinction coefficients of PAP^{3-} and $PAPH^{2-}$, respectively, and $K_a' = [PAP^{3-}][H^+]/[PAPH^{2-}]$.

A value of 1520 was estimated for E_1 from Fig 1, as E_{observed} at a high pH, and $E_2 = 675$ was calculated from Equation 2, the experimental data obtained in imidazole buffer and the titration data in Table I being used. The theoretical curve calculated from Equation 2 corresponds well with the experimental points for E_{observed} in imidazole (Fig 1). This buffer has the advantage that no metals are introduced, and the slight difference in the data for the two buffers (Fig 1) is probably due to the presence of potassium in the phosphate buffer. Potassium shifts the

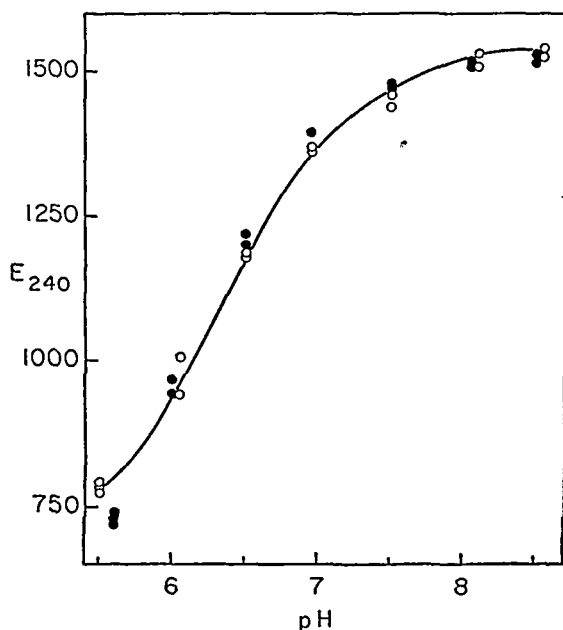


FIG 1 The effect of pH on the molar extinction coefficient at 240 $m\mu$ (E_{240}) of PAP in 0.05 M imidazole buffer (O) and in 0.05 M phosphate buffer (●). The theoretical curve (solid line) was calculated from Equation 2, the value pK_3' (PAP) = 6.35 from Table I being used.

pK_a of PAP and also decreases the extinction coefficient of PAP^{3-} , as do magnesium and other metals as well (Fig 2). The variations of E_{observed} with pH in a solution containing constant concentrations of potassium and magnesium ions were determined, and the data obtained for the effect of pH on the kinetics and the equilibrium of the enolase reaction could be corrected for the extinction coefficient variation under the same conditions. Such a standard curve for the conditions for optimal enolase activity is shown in Fig 3.

Equilibrium of Enolase Reaction—In their original work on the isolation of enolase, Warburg and Christian (1) showed that magnesium, manganese, and zinc activate enolase, and more recently (4) iron has been

added to this list. Other metals such as beryllium, calcium, strontium, cadmium, cobalt, and nickel have been reported to be inactive (4) and

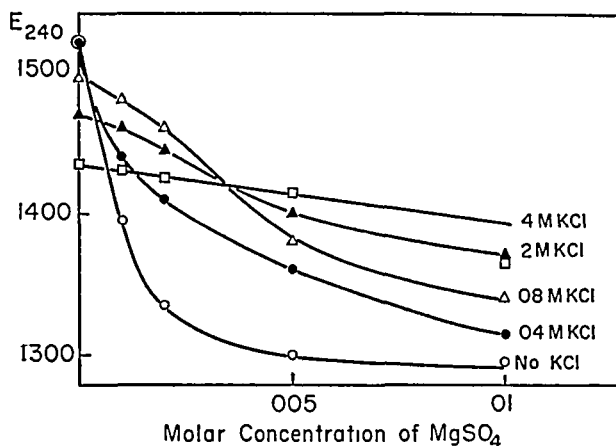


FIG 2 The effect of magnesium and potassium on the molar extinction coefficient at 240 $\text{m}\mu$ (E_{240}) of PAP in 0.05 M imidazole buffer at pH 8.35

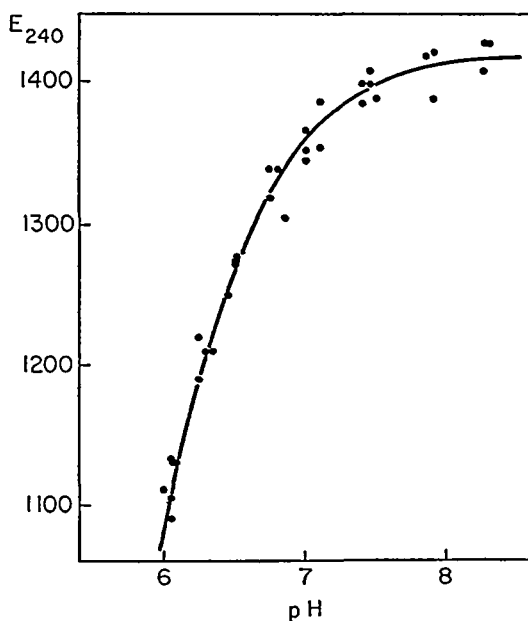


FIG 3 A correction curve for the effect of pH on the molar extinction coefficient at 240 $\text{m}\mu$ (E_{240}) of PAP in the standard assay medium: 0.008 M MgSO_4 , 0.4 M KCl, and 0.05 M imidazole buffer

also inhibitory when added to the activated enolase system (4, 12, 13). As has already been pointed out (4), a metal with less activating effect than magnesium will act as an inhibitor when added to the magnesium-

activated system. The reports in the literature of metals being inhibitory to the magnesium-activated enolase reaction should, therefore, not be interpreted to mean that these metals cannot activate the enzyme in the absence of magnesium.

In this work a total of 6 divalent metal ions was found to activate enolase, namely magnesium, manganese, zinc, cadmium, cobalt, and nickel. Iron was not tested. The reaction rate in the presence of cobalt and nickel was very low compared to that in the presence of the other metals, but was still significantly higher than the rate in the absence of metals. The relative activation strength of the different metals is discussed in Paper II (14) of this series.

The effects of pH and metal ions on the equilibrium of biochemical reactions have been discussed by several workers (15-19). If a reaction between two acids, AH and BH , is affected by the hydrogen ion concentration or the metal ion concentration, the over-all apparent equilibrium constant is expressed by

$$K_{\text{apparent}} = \frac{[B]_{\text{total}}}{[A]_{\text{total}}} = \frac{[B^-] + [BH] + [BM]}{[A^-] + [AH] + [AM]} \quad (3)$$

Here the different concentrations can be expressed in terms of the respective ionization constants (K_a') and binding constants (D')

$$[AH] = \frac{[H^+][A^-]}{K_{aA}'}, \quad [BH] = \frac{[H^+][B^-]}{K_{aB}'}$$

$$[AM] = D_A'[M][A^-], \quad [BM] = D_B'[M][B^-]$$

and substituting these values in Equation 3, one obtains

$$K_{\text{apparent}} = \frac{[B^-]}{[A^-]} \times \frac{1 + [H^+]/K_{aB}' + D_B'[M]}{1 + [H^+]/K_{aA}' + D_A'[M]}$$

The quantity $[B^-]/[A^-]$ is a constant and represents the pH- and metal-independent equilibrium constant K_{eq} , and Equation 3 can thus be written

$$K_{\text{apparent}} = K_{\text{eq}} \frac{1 + [H^+]/K_{aB}' + D_B'[M]}{1 + [H^+]/K_{aA}' + D_A'[M]} \quad (4)$$

If more than one metal is involved, the more general form of Equation 4 can be used

$$K_{\text{apparent}} = K_{\text{eq}} \frac{1 + [H^+]/K_{aB}' + D_{B_1}'[M_1] + D_{B_2}'[M_2] + \dots + D_{B_n}'[M_n]}{1 + [H^+]/K_{aA}' + D_{A_1}'[M_1] + D_{A_2}'[M_2] + \dots + D_{A_n}'[M_n]} \quad (5)$$

where D_1 , D_2 , and D_n are the binding constants for the complexes of the substrates A and B with the metals M_1 , M_2 , and M_n

It was found early in this work that the apparent equilibrium constant for the enolase reaction, expressed as $K_{\text{apparent}} = [\text{PAP}]/[\text{GA2P}]$, varied with both the metal concentration and pH. These variations are shown in Figs 4 to 6. In order to obtain the metal- and pH-independent equilibrium constant, the experimental values for K_{apparent} , and the ionization and

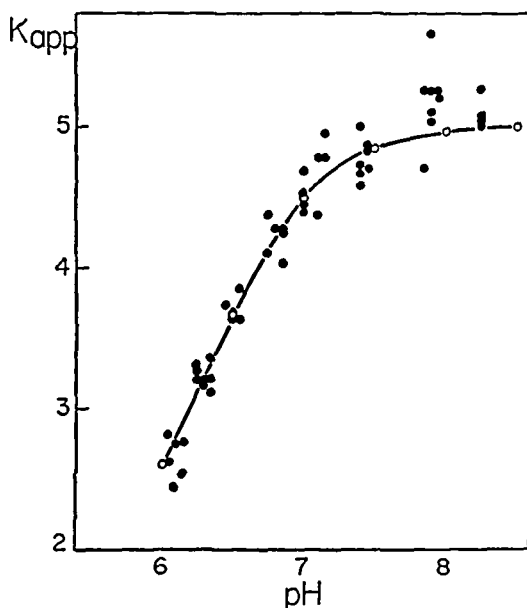


FIG 4 The variation of the apparent equilibrium constant for the enolase reaction with pH in a medium containing 0.008 M MgSO_4 , 0.4 M KCl , and 0.05 M imidazole buffer. The theoretical curve (O) was calculated from Equation 5 with the following data from Tables I and II: $\text{p}K_3'(\text{GA2P}) = 7.1$, $\text{p}K_3'(\text{PAP}) = 6.4$, $D_{K'}(\text{GA2P}) = 15$, $D_{K'}(\text{PAP}) = 12$, $D_{Mg'}(\text{GA2P}) = 280$, $D_{Mg'}(\text{PAP}) = 180$, and led to the value 6.3 for the pH- and metal-independent equilibrium constant, K_{eq} .

binding constants from Tables I and II, were substituted into the proper form of Equation 5. In this way K_{eq} was found to be 6.3.

The solid and broken lines in Figs 4 to 6 represent the theoretical curves calculated from Equation 5, by use of the value 6.3 for K_{eq} and the data in Tables I and II for the $\text{p}K_a'$ values and the binding constants. They coincide quite well with the experimental points. All the calculations were based on an uncertainty of 0.05 in the determination of the $\text{p}K_a'$ values. Bock and Alberty (16) have shown the effect of a variation of this magnitude on the calculated equilibrium constants, and it is obvious that the method is sensitive to titration errors. It is felt, however, that Equation 5 adequately describes the enolase equilibrium.

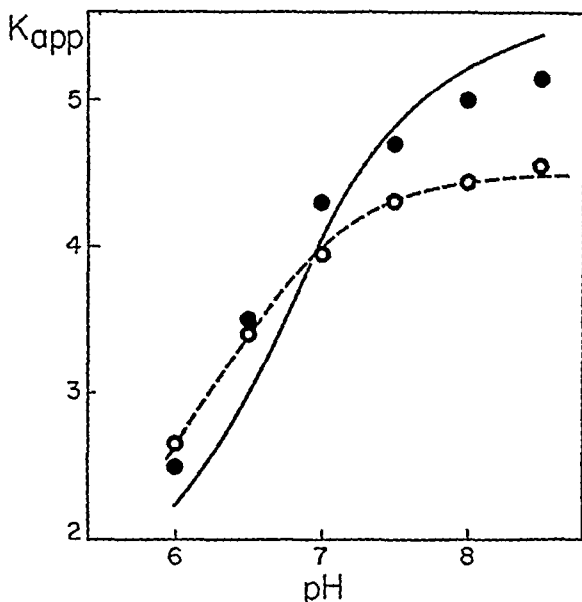


FIG 5 The variation of the apparent equilibrium constant for the enolase reaction with pH at two magnesium concentrations (●, 0.001 M MgSO_4 , ○, 0.01 M MgSO_4) in 0.05 M imidazole buffer. The corresponding theoretical curves were calculated from Equation 5 using the following data from Tables I and II: pK_3' (GA2P) = 7.0, pK_3' (PAP) = 6.35, D_{Mg}' (GA2P) = 280, D_{Mg}' (PAP) = 180, and led to the value 6.1 for K_{eq} . No attempt was made to correct for the change in ionic strength.

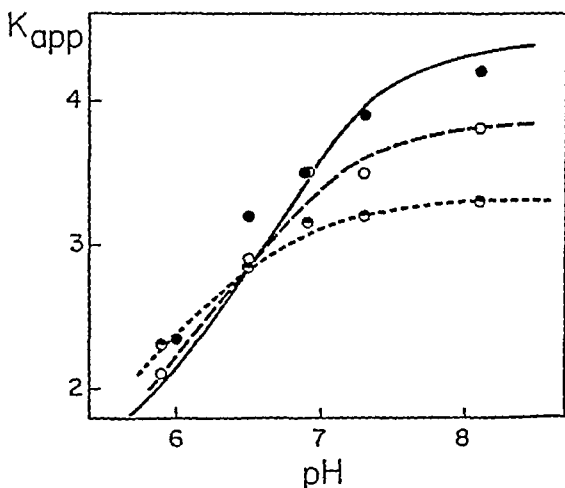


FIG 6 The variation of the apparent equilibrium constant for the enolase reaction with pH at three manganese concentrations (●, 0.001 M MnSO_4 , ○, 0.002 M MnSO_4 , ◐, 0.005 M MnSO_4) in 0.05 M imidazole buffer. The corresponding theoretical curves were calculated from Equation 5 using the same pK' values as in Fig 5 and the binding constants D_{Mn}' (GA2P) = 1225 and D_{Mn}' (PAP) = 560 from Table II, and led to the value 6.3 for K_{eq} . No attempt was made to correct for the change in ionic strength.

The equilibrium constants for the enolase reaction reported in the literature vary between 1.4 and 3.9 (1, 20-23), but are all apparent constants, dependent on metal concentration and pH. The higher value for the metal- and pH-independent equilibrium constant determined in this work is, therefore, not in conflict with the older data.

The graphical picture, obtained for different concentrations of magnesium and manganese (Figs 5 and 6), was also obtained for zinc, cadmium, cobalt, and nickel, and is also the same as Tievelyan *et al* (17) found for phosphorylase. It is interesting to note that for each metal there is a pH at which the apparent equilibrium constant is independent of the metal concentration. Utter and Werkman (12) showed that the over-all equilibrium between GA3P and PAP, involving both glyceric acid phosphate mutase and enolase, is independent of the metal concentration. If their study were conducted at, or close to, the pH of the isosbestic point, this could account

TABLE IV
*Effect of Temperature on Apparent Equilibrium Constant
for Enolase Reaction*

Determined in 0.05 M imidazole buffer containing 0.4 M potassium chloride and 0.008 M magnesium sulfate at pH 7.5

°K	K_{apparent}	ΔH , calories per mole
288.0	3.54	3320
299.5	4.55	3700
307.5	5.28	

for their observation. Another possibility is that the metal binding to GA3P and PAP is equal, which in itself has interesting thermodynamic implications.

With the new equilibrium constant, $K_{\text{eq}} = 6.3$, ΔF_{298} for the enolase reaction was found to be -1090 calories per mole, and from the thermal data in Table IV the average value of 3500 calories per mole was obtained for ΔH_{298} . It should be noted that the latter value is not independent of the metal concentration and pH.

SUMMARY

The preparation of the cyclohexylammonium salt of enolpyruvic acid phosphate has been described, and some of its properties as a substrate for enolase have been discussed.

From the study of the equilibrium of the enolase reaction it has been shown that both metal ions and hydrogen ions are involved in the over-all stoichiometry of the interconversion of D-glyceric acid 2-phosphate and

enolpyruvic acid phosphate A mathematical expression for the theoretical variations of the apparent equilibrium constant with pH and metal concentration has been derived, and from the determination of the pK_a' values and the metal-binding constants of the substrates, the pH- and metal-independent equilibrium constant has been evaluated and found to be 6.3

A total of 6 divalent metal ions, magnesium, manganese, zinc, cadmium, cobalt, and nickel, was found to activate enolase, and the effect of all of these metals on the equilibrium of the enolase reaction has been determined

The new value of the equilibrium constant and studies of the effect of temperature on the equilibrium have yielded the following thermodynamic constants $\Delta F_{298} = -1090$ calorie per mole and $\Delta H_{298} = 3500$ calories per mole

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STUDIES ON THE ENZYME ENOLASE*

II KINETIC STUDIES

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The philosophy behind much of the recent research in enzymology is that the activity of enzymes can be related to specific groups in the enzyme polypeptide chain, and the concept of the "active site" of an enzyme may soon become a reality. Kinetic studies of enzymes such as fumarase (1-3), acetylcholinesterase (4-6), arginase (7), alcohol dehydrogenase (8), and others have led to formulations of specific reaction mechanisms and of models of the active sites. Preliminary studies of enolase (9, 10) indicated that this enzyme is suitable for similar research. The enzyme has been crystallized (11), both the substrates are available as pure, stable, water-soluble salts (9, 10), and the equilibrium of the reaction makes possible investigation of both the forward and the reverse reaction. Malmstrom (12-16) has studied the effects of various metals in activating enolase and has arrived at certain conclusions as to the active site and the mechanism of the dehydrating action of the enzyme.

In the present paper, the results of some further kinetic studies of the enolase reaction will be discussed in relation to a possible model of the enzyme and its mechanism of action. The nature of the substrate inhibition first described by Malmstrom (12) is clarified (14). The substrate specificity is outlined from a study of several substrate analogues, and some information concerning the nature of the ionizing groups in the active site of the enzyme has been obtained from the pH activation curves and group-specific reagents.

EXPERIMENTAL

Materials and Methods—The materials and the general methods were discussed in Paper I of this series (10). The enzyme activity was determined by the method of Warburg and Christian (11), based on the ultra-

* This work was supported by grants from Eli Lilly and Company and the Cancer Research Funds of the University of California. The experimental data in this paper are taken from the thesis submitted by Finn Wold to the Graduate Division of the University of California in September, 1956, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

† Du Pont Postgraduate Fellow in Biochemistry, 1955-56.

violet absorption of enolpyruvic acid phosphate¹ (10) The measurements were made at 240 m μ by use of a Beckman DUR spectrophotometer connected to a Brown recording potentiometer (strip chart) through a specially constructed amplifier, allowing either 0 to 100 per cent or 90 to 100 per cent transmission to be recorded on full scale width (17) In this work, the most sensitive scale (90 to 100 per cent transmission or 0.0458 to 0 optical density unit) was used In all experiments the velocity of only the first 5 or 10 per cent of the reaction was recorded This allowed the determination of the initial velocities in terms of optical density change per unit of time directly from the slope of the tracings

The experimental procedure for the rate measurements was to prepare 3 ml samples of the desired reaction mixture in the cuvette and set the instrument to zero A small volume (0.02 to 0.05 ml) of enzyme was then added from a small Teflon cup, which was also used simultaneously to mix the contents of the cuvette The chart motor was started at the moment the enzyme was introduced, and the spectrophotometer was turned on as soon as the enzyme addition was completed In this way a time interval of only 5 to 7 seconds elapsed between the addition of the enzyme and the start of the recording

The kinetic measurements were carried out in imidazole buffer This buffer has several advantages over those previously used In addition to its favorable pK (6.8), its suitability is based on the facts that no metal is introduced with the buffer and that the buffer itself is relatively inert in the enzymatic reaction As shown below, phosphate inhibits the enolase reaction, and the rates obtained in the presence of imidazole are much higher than those in the presence of phosphate, tris(hydroxymethyl)aminomethane, or bicarbonate (18)

The kinetic constants were estimated from Lineweaver-Burk and Dixon plots (19, 20), either directly from the plots or according to the method of the least squares

Due to the optimal rates of the reaction, the following medium was chosen as a basis for the variations studied in this work 0.05 M imidazole buffer, 0.008 M magnesium sulfate, 0.4 M potassium chloride, and approximately 10^{-4} M substrate

RESULTS AND DISCUSSION

Enzyme Specificity and Effect of Substrate and Substrate Analogues—Of all the compounds tested in this work² (Table I), only GA2P and PAP were

¹ The following abbreviations and symbols are used GA2P, glyceric acid 2-phosphate, GA3P, glyceric acid 3-phosphate, PAP, enolpyruvic acid phosphate, v , steady state reaction velocity, V , maximal initial velocity, K_s , Michaelis constant, and K_i , inhibition constant

² Prepared in this laboratory by procedures to be published elsewhere

found to be substrates for enolase. The synthetic analogue of GA2P, *D-erythro*-2,3-dihydroxybutyric acid 2-phosphate, gave no evidence of dehydration, *i e* no increase in ultraviolet absorption, after prolonged incubation with the enzyme. Enolase thus appears to be specific for its

TABLE I
*Compounds Tested As Inhibitors for Enolase Forward
Reaction under Standard Conditions at pH 7.8*

Compound	Inhibition	K_i (mole per liter)
D-Lactic acid	None	
D-Glyceraldehyde 3-phosphate	"	
Dihydroxyacetone phosphate	"	
β -Glycerol phosphate	"	
β -Hydroxypropionic acid phosphate	Competitive	4.5×10^{-4}
D-Lactic acid phosphate	"	3.5×10^{-4}
D-Glyceric acid 3-phosphate	"	4.5×10^{-4}
D-erythro-2,3-Dihydroxybutyric acid 2-phosphate	"	6.0×10^{-4}
D-erythro-2,3-Dihydroxybutyric acid 3-phosphate	"	3.3×10^{-3}
Inorganic phosphate	Non-competitive	6.4×10^{-3}

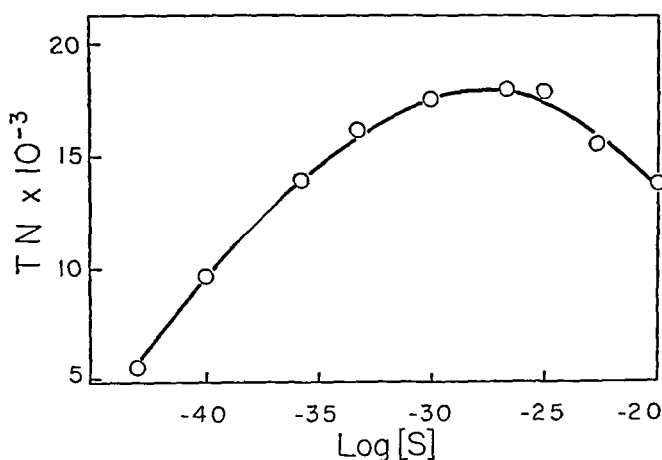


FIG 1 The effect of substrate concentration on the rate of the enolase reaction. The system consisted of 0.008 M MgSO_4 and 0.05 M imidazole buffer at pH 7.0. T N = turnover number.

natural substrates. A more definite conclusion awaits synthesis of *L-threo*-2,3-dihydroxybutyric acid 2-phosphate, which may be acted upon by the enzyme (see the formulas below).

The effect of the substrate concentration on the rate of the enolase reaction is shown in Fig 1, and the inhibition at high substrate concentration is apparent. The substrate inhibition was first reported by Malmström

violet absorption of enolpyruvic acid phosphate¹ (10) The measurements were made at 240 m μ by use of a Beckman DUR spectrophotometer connected to a Brown recording potentiometer (strip chart) through a specially constructed amplifier, allowing either 0 to 100 per cent or 90 to 100 per cent transmission to be recorded on full scale width (17) In this work, the most sensitive scale (90 to 100 per cent transmission or 0.0458 to 0 optical density unit) was used In all experiments the velocity of only the first 5 or 10 per cent of the reaction was recorded This allowed the determination of the initial velocities in terms of optical density change per unit of time directly from the slope of the tracings

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Dihydroxyacetone phosphate	"	
β -Glycerol phosphate	"	
β -Hydroxypropionic acid phosphate	Competitive	4.5×10^{-4}
D-Lactic acid phosphate	"	3.5×10^{-4}
D-Glyceric acid 3-phosphate	"	4.5×10^{-4}
<i>D-erythro</i> -2,3-Dihydroxybutyric acid 2-phosphate	"	6.0×10^{-4}
<i>D-erythro</i> -2,3-Dihydroxybutyric acid 3-phosphate	"	3.3×10^{-3}
Inorganic phosphate	Non-competitive	6.4×10^{-3}

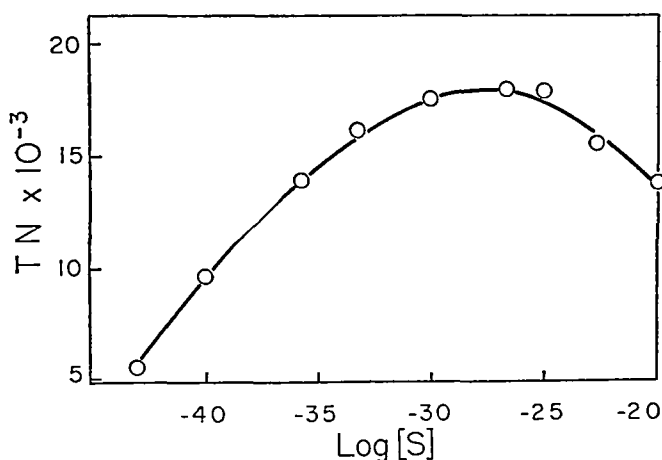


Fig. 1. The effect of substrate concentration on the rate of the enolase reaction. The system consisted of 0.008 M MgSO_4 and 0.05 M imidazole buffer at pH 7.0. T N = turnover number.

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The effect of the substrate concentration on the rate of the enolase reaction is shown in Fig. 1, and the inhibition at high substrate concentration is apparent. The substrate inhibition was first reported by Malmström

(12), who, working with DL-GA2P, suggested that the inhibition was due to the unnatural L isomer. Since the pure D-GA2P used in this work gave the same inhibition, this hypothesis is not tenable. More recently Malmstrom and Westlund (16) proposed that the inhibition is due to the binding, and thus the removal of the activating metal ion by the substrate. The experimental evidence obtained in our work (Table II) is inconsistent with this hypothesis also. If the substrate inhibition were due to removal of metal by substrate, the inhibition should occur at lower substrate concentration when the metal concentration was lowered, which is clearly not the case. When it was found that the totally ionized species GA2P^{3-} and PAP^{3-} appear to be the active substrates for enolase (see below), the possibility was considered that other ionic species of the substrates (e.g. GA2PH^{2-} and PAPH^{2-}) might combine with the enzyme and cause inhibi-

TABLE II

Combined Effects of Substrate, Magnesium, and pH on Rate of Enolase Reaction in 0.05 M Imidazole Buffer

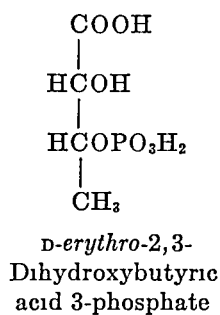
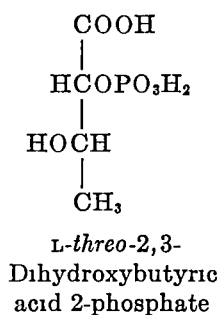
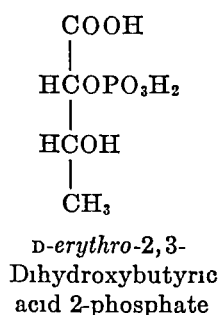
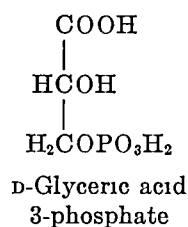
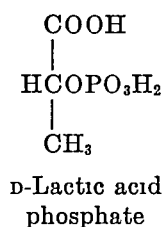
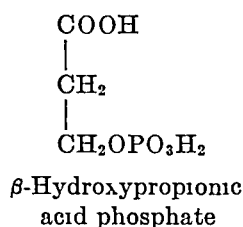
Concentration of Mg	pH	Rates (Δ optical density per min.) at indicated substrate concentrations					
		1 M	2 M	5 M	10 M	50 M	100 M*
M							
0.008	7.5	0.078	0.085		0.082	0.080	
0.008	6.0	0.039	0.053		0.055	0.054	
0.008	7.1		0.057	0.068	0.068	0.068	0.055
0.001	7.1		0.069	0.069	0.072	0.066	0.057

* Molar substrate concentrations $\times 10^4$

tion. The data in Table II also appear to exclude such a mechanism, since the substrate concentration causing inhibition does not decrease with the pH. The most probable explanation of the substrate inhibition is that the active substrate combines with a neighboring site on the enzyme and thereby decreases the rate of breakdown of the active enzyme-substrate complex. Alberty (21) has discussed such a mechanism, which is consistent with the data on the substrate inhibition of fumarase (2), urease (22), and acetylcholinesterase (4). The general form of the substrate inhibition curve for enolase fits this mechanism.

In an attempt to establish the chemical constitution required for a compound to combine with the active site of enolase, several compounds were tested as inhibitors in the forward reaction. The results (Table I) show that, of the organic phosphates tested, only those with both a carboxyl and a phosphate group caused inhibition. The spatial separation of the carboxyl and the phosphate groups appears to have some influence on the

binding, lactic acid phosphate being slightly more strongly bound than both β -hydroxypropionic acid phosphate and GA3P. The effect of replacing a hydrogen of the natural substrate with a methyl group is shown by the higher value of K , for *D-erythro*-2,3-dihydroxybutyric acid 2-phosphate. The combined effect of replacing a hydrogen with a methyl group and separating the carboxyl from the phosphate group is illustrated by the fact that *D-erythro*-2,3-dihydroxybutyric acid 3-phosphate is bound only one-tenth as strongly as is lactic acid phosphate.



Effect of pH—All of the reaction velocities, measured in terms of optical density change per unit of time, were corrected for the effect of pH and metals on the molar extinction coefficient of PAP (10), so that throughout this work change in optical density is equivalent to change in molar concentration of GA2P or PAP.

The pH optimal curve for the forward reaction is given in Fig. 2. In further studies of the pH effects, the variations in V and K_s with pH were determined for both the forward and the reverse reaction. The plot of V against pH is shown in Fig. 3. Since V (equal to $k_3[E]_0$) is independent of the substrate concentration, any change in V must signify a change in the enzyme-substrate complexes. A comparison of the curves in Figs. 2 and 3 shows that the increase in the rate in the first part of the activity curve in Fig. 2 is due to ionization of the substrate (V is independent of pH in Fig. 3), and hence that the totally ionized species of GA2P and PAP are the true substrates for enolase. On the other hand, the decrease in

both v and V in the last part of the pH curves is due to changes in the enzyme

To test the reversibility of the pH inactivation, the following experiment was conducted. The pH of a standard reaction mixture of imidazole, KCl,

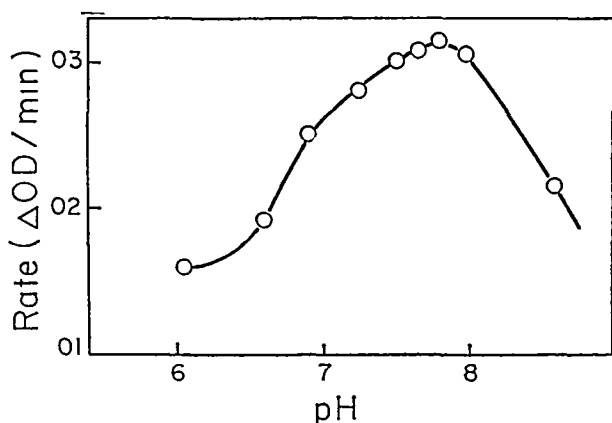


FIG 2 The effect of pH on the rate of the enolase reaction. The system consisted of 0.008 M MgSO_4 , 0.4 M KCl, 0.05 M imidazole buffer, and 2×10^{-4} M GA2P.

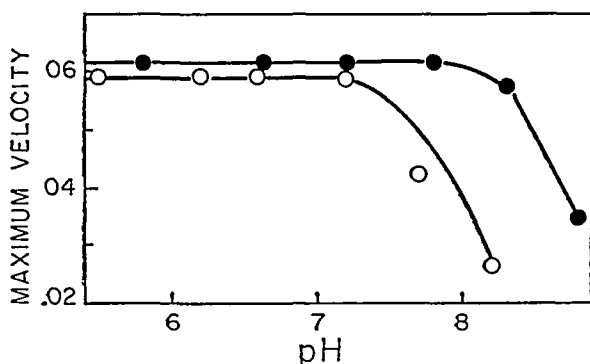


FIG 3 The effect of pH on the maximal initial velocity for the forward (●) and the reverse (○) reaction. The system consisted of 0.008 M MgSO_4 , 0.4 M KCl, and 0.05 M imidazole buffer. The individual values of V were obtained from Lineweaver-Burk-Dixon plots. The two curves were obtained from different experiments, and do not represent the true relative magnitude of V for the forward and the reverse reaction.

MgSO_4 , and GA2P was adjusted to pH 10, and the amount of acid required to bring the pH of 3 ml aliquots of this reaction mixture to pH 8 was accurately determined. In the controls, the acid was added before the enzyme and the rate was determined at pH 8. In the experimental runs, the enzyme was added first and the rate at pH 10 was determined for a time interval of about 2 minutes. The acid was then added, and the new rate for the same sample at pH 8 was obtained. The results are given in

Table III They show that the inactivation up to pH 10 is reversible, and thus exclude irreversible denaturation as a cause of the inactivation

The possibility that the inactivation is due to removal of magnesium as insoluble $Mg(OH)_2$ can also be tested The solubility product of $Mg(OH)_2$ is 1.2×10^{-11} at 18° (23) For a 0.008 M solution of Mg^{++} , the ion product will exceed the solubility product at pH values above 9.6, but below this pH no $Mg(OH)_2$ will precipitate It has been suggested, however, that hydrated ionic species of an activating metal may combine with the enzyme and cause inhibition (24)

Assuming that the pH inactivation is due to titration of an active group in the enzyme, one can estimate the pK' of this active group as the pH value at which V is one-half of its maximal value (corresponding to the point where the active enzyme is reduced to one-half of its original concen-

TABLE III
Demonstration of Reversibility of pH Inactivation of Enolase

Experiment No	Rate (Δ optical density per min) at pH 10	Rate (Δ optical density per min) at pH 8
Control		0.158
1	0.020	0.154
2	0.021	0.144
3	0.014	0.130

tration) In this way, the values 8.9 and 8.1 were obtained for the enzyme complexes of GA2P and PAP, respectively (Fig. 3)

The theoretical titration curves calculated from the pK' values above do not coincide exactly with the experimental points in Fig. 3 The total effect of pH on V thus seems to be due to several factors such as titration of an active group in the enzyme-substrate complex and the inhibition caused by the combination of the enzyme with $MgOH^+$ and $Mg(OH)_2$

In a further attempt to measure the dissociation constant for the active site in the enzyme, the negative logarithm of the Michaelis constant (pK_s) was plotted against pH according to the method of Dixon (25) Fig. 4 gives the resulting curves for the forward and reverse reaction, showing breaks at pH 7.4 and 7.6, respectively If the average, 7.5, is taken to be the dissociation constant for a group in the active site of enolase, the values 8.1 and 8.9 determined for the two enzyme-substrate complexes correspond to the expected acid-weakening or base-strengthening effect of the substrate anions in close proximity to the dissociating group This effect should be directly comparable to the basicity of the anion, and the difference of units of pH 0.8 between the two pK' values in Fig. 3 is in accord

with the difference in the titration values of the free substrates (pK_3' (GA2P) = 7.1 and pK_3' (PAP) = 6.4 (10)). A similar effect of the substrates on the ionization of fumarase has been reported (1).

Effect of Group-Specific Protein Reagents—The most common groups in proteins that have a pK of 7.5 are α -amino groups, imidazolium groups, and sulfhydryl groups (26, 27). In an attempt to distinguish between these three possible groups in the active site of enolase, certain reagents, the specificities of which have been fairly well established, were tested as inhibitors. The enzyme was incubated with approximately equal concentrations of the different inhibitors, and its activity was tested at a single

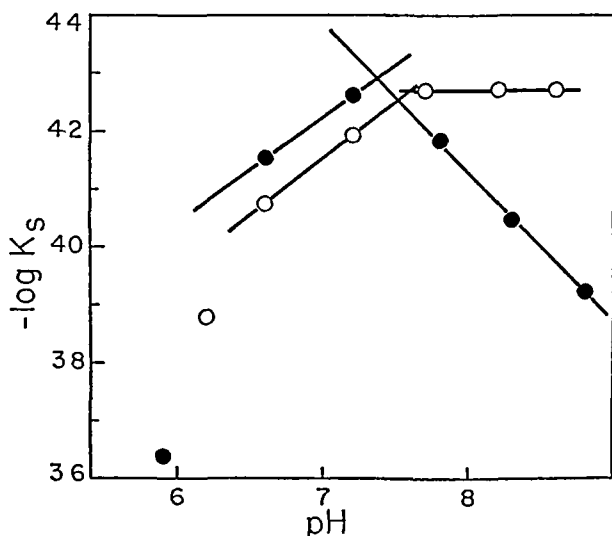


FIG. 4 The variation of the negative logarithm of the Michaelis constant for the forward (●) and the reverse (○) reaction. The K_s values were obtained from the same experiments and plots as the V values in Fig. 3.

substrate concentration. In order to avoid buffer interference with the imidazole reagents, this experiment was run in phosphate buffer. The results are shown in Table IV and are as conclusive as these data can be in eliminating sulfhydryl as part of the active site of enolase. The preliminary nature of this experiment and the lack of definite knowledge of the specificity of the other inhibitors used (28–30) make it difficult to interpret the rest of the results in Table IV. The fact that diisopropyl phosphorofluoridate and *p*-nitrobenzoyl chloride inhibit enolase is in itself interesting, and a more careful and extensive study of the inhibition of enolase by different protein reagents is planned.

Studying the effect of pH on the binding of zinc to enolase, Malmstrom (16) came to the conclusion that the metal is bound to a group with a pK'

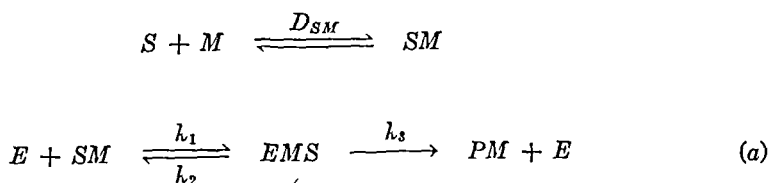
around 6 and suggested that this group may be imidazolium. This is not in conflict with the data obtained here. If the metal is bound to an imidazolium group ($pK' = 6$), the ionization of this group would not be detectable by kinetic studies conducted in the presence of optimal concentrations of metal. The active site of enolase thus appears to contain one imidazolium group ($pK' = 6$) which is involved in the binding of the activating metal, and, in addition, another imidazolium group or an α -amino group ($pK' \sim 7.5$) which is involved in direct interaction with the substrates.

TABLE IV
Effect of Group-Specific Reagents on Enolase Reaction

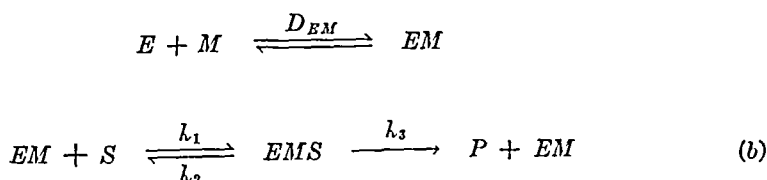
Reagent	Specificity for			Rate (Δ optical density per min)
	SH	NH ₂	Imidazole	
None				0.044
Iodoacetic acid	+			0.044
Iodoacetamide	+			0.044
<i>p</i> -Chloromercuribenzoate	+			0.041
Photooxidation	+		+	0.000
Diisopropyl phosphorofluoridate*	+		+ (?)	0.000
<i>p</i> -Nitrobenzoyl chloride			+ (?)	0.003
Formaldehyde	?	+	?	0.022

* Diisopropyl fluorophosphate (DFP)

Mechanism of Metal Activation—The two simplest general mechanisms for metal activation are



and



The steady state treatment will yield the following rate laws

$$v = \frac{V}{1 + K_s/[S] + K_s/D_{SM}[M][S]} \quad (1)$$

$$v = \frac{V}{1 + K_s/[S] + K_s/D_{EM}[M][S]} \quad (2)$$

corresponding to mechanisms (a) and (b), respectively. These equations are derived from the assumption that the metal concentration is much higher than the concentration of both the substrate and the enzyme.

The only difference between Equations 1 and 2 is that one contains the binding constant for the metal-substrate complex and the other that for the metal-enzyme complex. In general, kinetic data cannot distinguish between these two mechanisms (2).

Warburg and Christian (11) originally showed that the kinetics of the metal activation of enolase could be explained by mechanism (b). More recently, Malmstrom (12-16) has arrived at the same conclusion by elaborate studies of the activation kinetics and of the binding of the activating metal to the enzyme and to the substrates. In view of the rather high concentrations of magnesium required for the activation of enolase, however, it appears that mechanisms (a) and (b) can be distinguished by the following, simple experiment. If the variations in v with varying metal concentration are plotted according to Lineweaver-Burk-Dixon ($1/v$ against $1/[M]$), Equation 1 yields a straight line with intercept on the $1/[M]$ axis equal to

$$1/[M] = D_{SM} (1 + [S]/K_s)$$

Similarly, Equation 2 gives a $1/[M]$ intercept of

$$1/[M] = D_{EM} (1 + [S]/K_s)$$

If the substrate concentration is held constant and equal to K_s , mechanisms (a) and (b) in an experiment like this will yield $1/[M]$ intercepts equal to $2D_{SV}$ and $2D_{EM}$, respectively. Regardless of the numerical values of the respective binding constants, it is obvious that the forward and the reverse reaction for mechanism (a) will give intercepts differing by a factor corresponding to the difference in the magnesium-binding constants of GA2P and PAP, which are 280 and 180, respectively (10), whereas the same plots for mechanism (b) must give a common intercept, since the binding of the metal to the enzyme according to mechanism (b) is the same for the forward and the reverse reaction. This reasoning is based on the assumption that K_s is constant over the range of metal concentrations

used This assumption was tested for the forward reaction, and the results are shown in Table V Since the metal range used in the experiments was from 1×10^{-3} M to 5×10^{-3} M, the above assumption holds The intercepts for the forward and the reverse reaction are identical (Fig 5) This is fairly conclusive in eliminating mechanism (a), the combination of the metal with the substrate It does not mean that mechanism (b), the

TABLE V

Variations of V and K_s for Forward Reaction with Varying Magnesium Concentration

Concentration of magnesium	V (Δ optical density per min)	K_s (moles per liter)
8×10^{-4}	0.104	1.09×10^{-4}
5×10^{-3}	0.174	1.04×10^{-4}
2×10^{-2}	0.124	1.20×10^{-4}

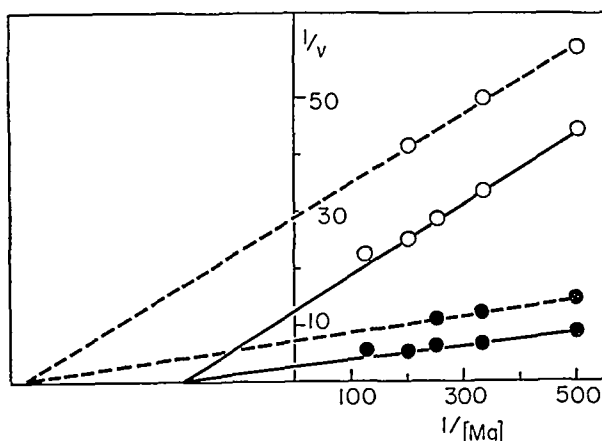


FIG 5 Lineweaver-Burk-Dixon plot of $1/v$ against $1/[Mg]$ for the forward (●) and the reverse (○) reaction, in the absence of inhibitor (solid lines), and in the presence of 4×10^{-5} M $MnSO_4$ (dotted lines) The system consisted of 0.05 M imidazole buffer at pH 7.15, 0.4 M KCl, 1×10^{-4} M GA2P for the forward reaction, and 2×10^{-4} M PAP for the reverse reaction

simple combination of the metal with the enzyme, is correct, although the evidence presented here is as conclusive as that presented previously for enolase (11–16) and for other enzymes as well (31–34) In addition, the “non-competitive activation” of enolase by suboptimal metal concentrations (Table V) favors the proposition that it is primarily the enzyme that is involved in the metal activation

Metal Inhibition—The inhibition by metal excess (Table V) is predominantly non-competitive, indicating an inactivation of the enzyme

rather than the formation of an inactive metal-substrate complex. The mechanism may be similar to that proposed for the substrate inhibition, namely that at high metal concentrations a combination of a second metal ion with the enzyme results in inactivation. Such a mechanism is in agreement with the one proposed by Malmstrom (13), and does also fit the proposed picture of the active site of enolase. If there are two imidazole groups in the active site, one binding the metal and the other interacting directly with the substrates, the metal inhibition could arise from a combination of metal to both the imidazole groups.

TABLE VI
Some Properties of Common Divalent Metals (23, 37)

Metal	Ionic radius	Electronegativity*	Enolase activity
Be	0.34	1.5	-†
Mg	0.78	1.2	+
Ca	1.06	1.0	-
Mn	0.62	1.4	+
Fe(II)	0.83	1.65	+†
Co	0.82	1.7	+
Ni	0.78	1.7	+
Cu(II)	0.70	2.0	-†
Zn	0.83	1.5	+
Sr	1.27	1.0	-
Cd	1.03	1.5	+
Ba	1.43	1.1	-
Hg(II)	1.12	1.9	-
Pb	0.84	1.6	-

* For definition of a unit, see Coryell (37)

† From the data of Malmström (15)

Malmstrom (15) tested some of the inactive divalent metals as inhibitors for the magnesium-activated enolase, and found them to be competitive inhibitors with respect to the activating metal. To test the inhibitory effect of a metal, which can itself activate the enzyme, we have studied the effect of manganese on the magnesium-activated system. Fig. 5 shows the $1/v$ against $1/[M]$ plots in the absence and in the presence of manganese, for the forward and reverse reaction, and the somewhat unexpected picture of an uncompetitive inhibition.

Comparative Effects of Activating Metals—Some of the properties of active and non-active divalent metals are compared in Table VI. It is clear that no single parameter can explain the effectiveness of the metals activating enolase.

Schubert (35) and Williams (36) have discussed the factors contributing

to the stability of complexes between metals and small organic molecules and polymers. Schubert has also pointed out that, regardless of the nature of the ligand or whether the metal is attached to oxygen, nitrogen, or sulfur, some general order exists with respect to the relative stability of the complexes of a series of metals, listed according to decreasing complex stability, as in the following Series A

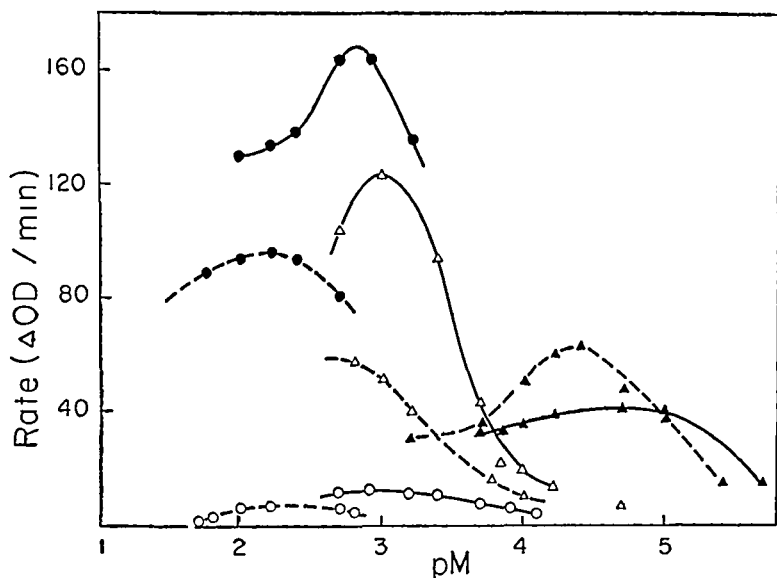
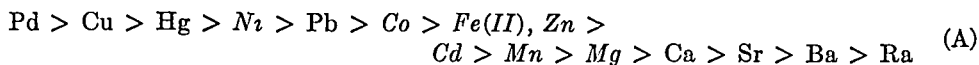
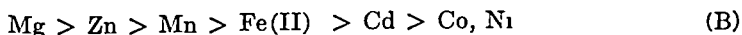


FIG 6 The relative effectiveness of some metals in activating enolase: magnesium (●), manganese (▲), zinc (△), and cadmium (○). The system consisted of 0.05 M imidazole buffer at pH 7.2 and 5×10^{-4} M GA2P in the absence of KCl (solid lines) and in the presence of 0.4 M KCl (dotted lines). The activity obtained with cobalt and nickel was too low to record on this graph. $\text{pM} = -\log \text{metal concentration}$.

Apparently the stability decreases with decreasing electronegativity (increasing basicity) of the metals, and it is interesting that the metals that activate enolase (italicized in Series A above) all fall together in the middle of the series.

When the relative activation strength of the metals was tested in the enolase reaction (Fig 6), Series B was obtained (Malmstrom's data (15) were used to give Fe its appropriate place)

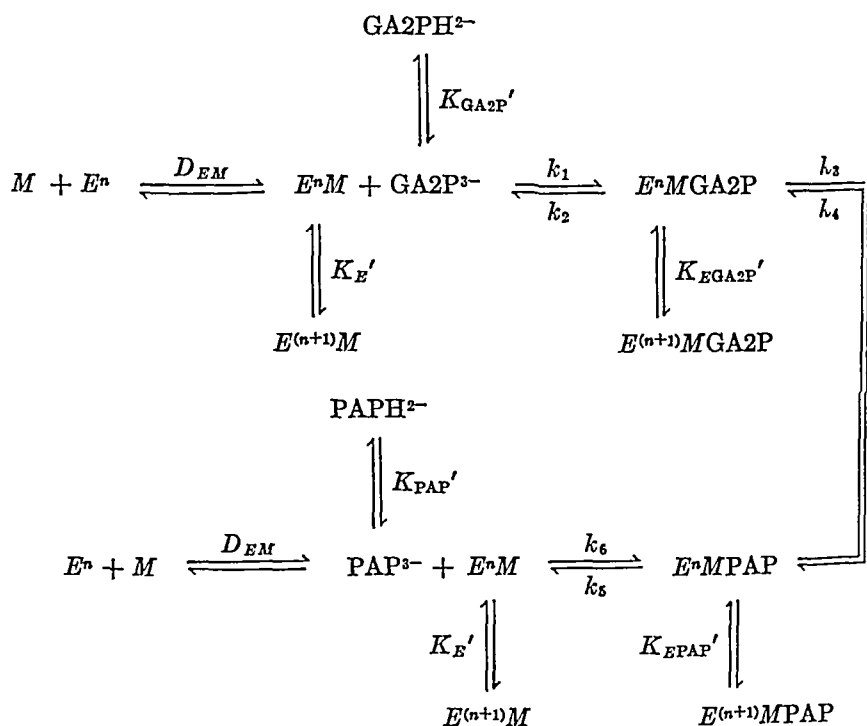


With the data in Table VI, the relative activation strength in Series B can be explained in terms of electronegativity of the metals if some allow-

ance is also made for solubility and ionic radius. Thus Ca, Sr, and Ba all have an electronegativity close to that of Mg, but their ionic radii are larger. On the other end of Series A, Pb is found with both an ionic radius and an electronegativity favorable to activity. Pb, however, forms insoluble complexes with both GA2P and PAP, and for that reason should appear to be inactive.

It is interesting to note the effect of the potassium ion on the relative effectiveness of the various metals in Fig. 6. The increase in the optimal metal concentration when potassium was added is in agreement with the expected decrease in the binding of the divalent metals in the presence of potassium. The total effect of potassium chloride is probably the sum of ionic strength effects and the specific effect on the binding of the activating metal ions, and elucidation will require further study.

A possible general mechanism for the enolase reaction is presented in the accompanying scheme.



In this scheme E^nM represents the active metal enzyme with n negative charges. $K_{E'}$, $K_{E\text{GA2P}'}$, $K_{E\text{PAP}'}$ are the dissociation constants for the ionizing group in the active center of the free metal enzyme ($\text{pK}_a' = 7.5$), in the metal enzyme-GA2P complex ($\text{pK}_a' = 8.9$), and in the metal enzyme-PAP complex ($\text{pK}_a' = 8.1$), respectively. $K_{\text{GA2P}'}$ and $K_{\text{PAP}'}$ are the

dissociation constants for the substrates (pK_a' values 7.1 and 6.4, respectively), and D_{EV} is the binding constant for the enzyme metal complex. This mechanism has not been rigorously tested, but is in accord with all the data presented above.

SUMMARY

Enolase appears to be specific for D-glyceric acid 2-phosphate (GA2P) and enolpyruvic acid phosphate (PAP), and the substrates must be in the totally ionized forms ($GA2P^{3-}$ and PAP^{3-}) for the reaction to take place. The enzyme is inhibited by high concentrations of the substrate, and the inhibition is most probably due to the combination of a second substrate molecule to a neighboring site on the enzyme.

A carboxyl group and a phosphate group are required for the interaction with the active site of the enzyme, and certain geometrical requirements with respect to the size of the molecule and to the separation of the carboxyl group and the phosphate group must also be satisfied.

Certain divalent metal ions are required for the enolase reaction, and the function of the metal ions presumably is to combine with the enzyme and thereby form the active metal enzyme.

A certain predictability as to which metals activate enolase is obtained from considerations of the electronegativities and the ionic radii of the metals and the solubilities of the different metal complexes.

The active site of enolase may contain an ionizable group, with a pK of about 7.5. The enzyme is reversibly inactivated when the group is titrated from its acidic to its basic form. This group appears to be either an imidazole or an α -amino group.

A general mechanism for the enolase reaction has been proposed.

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PYRIMIDINE METABOLISM

II ENZYMATIC PATHWAYS OF URACIL ANABOLISM*

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The ability of tissues to incorporate uracil, uridine, and uridine 5'-phosphate¹ into ribonucleic acid has been the subject of considerable investigation (1-4). The work of Plentl and Schoenheimer (5) had indicated that when rats are fed N¹⁵-uracil the label is not incorporated into the tissue RNA. Similar results were obtained by Rutman, Cantarow, and Paschakis when uracil-2-C¹⁴ was administered to rats (6). These authors also found that hepatomas induced by 2-acetylaminofluorene in rats, in contrast to normal liver, showed an extensive incorporation of uracil into RNA.

Experiments with C¹⁴-labeled uridine and UMP (7) have indicated that each of these is incorporated into the RNA of normal rat liver at a rate which is appreciably higher than that observed with uracil. Therefore, it was possible that the limited incorporation of uracil into the RNA of normal rat liver could be a reflection of an inefficient conversion of the base to the corresponding nucleoside or nucleotide.

The present investigation shows that there occurs in the normal rat liver a nucleoside phosphorylase capable of converting uracil to uridine in the presence of R-1-P, in addition, these studies demonstrate the existence in rat liver of uridine kinases which will catalyze the phosphorylation of uridine to UMP, UDP, and UTP. No evidence for the direct phosphoribosylation of uracil in the presence of PRPP has been found in rat liver, although the present investigation shows that this reaction will occur in some extracts of a uracil-requiring mutant of *Lactobacillus bulgaricus*. Accordingly, it is suggested that the inability of normal rat liver to incorporate uracil into RNA is not due to the absence of an anabolic pathway for

* This investigation was supported in part by grants from the American Cancer Society and from the National Institutes of Health, Public Health Service.

¹ The abbreviations used are RNA, ribonucleic acid, UMP, uridine 5'-phosphate, UDP, uridine 5'-diphosphate, UTP, uridine 5'-triphosphate, ADP, adenosine 5'-diphosphate, ATP, adenosine 5'-triphosphate, ITP, inosine 5'-triphosphate, R-1-P, ribose 1-phosphate, R-5-P, ribose 5-phosphate, PRPP, ribose 5-phosphate-1-pyrophosphate, Tris, tris(hydroxymethyl)aminomethane, EDTA, ethylenediaminetetraacetate, UDPG, uridine diphosphate glucose, TCA, trichloroacetic acid.

uracil, rather, it may be attributable, in part at least, to competition between the anabolic and the catabolic pathway, as will be shown in the discussion and in a subsequent paper of this series ²

EXPERIMENTAL

Materials and Methods—The preparation of the acetone powders of rat liver supernatant fraction used in these experiments has been described (8) The uracil-2-C¹⁴ and uridine-2-C¹⁴ were prepared from orotic acid-2-C¹⁴ Orotic acid-2-C¹⁴ was converted to UMP-2-C¹⁴ in the presence of PRPP and dialyzed crude bakers' yeast extract (9) This preparation will convert part of the UMP-2-C¹⁴ to uridine and uracil-2-C¹⁴, a circumstance presumably attributable to the presence of phosphatases and nucleosidases The uracil-2-C¹⁴, uridine-2-C¹⁴, and UMP-2-C¹⁴ were separated by ion exchange chromatography (10) and further purified by paper chromatography, with use of various solvent systems (11–13)

The extent of the reaction in the presence of nucleoside phosphorylase was followed by two methods In one, the appearance or disappearance of ribose was measured in the supernatant solution obtained after precipitation with TCA by using a modified orcinol test (14), in the other, used with the more purified enzyme fractions, the increase in absorption at 285 m μ coincident with the conversion of uridine to uracil in 1 N NaOH solution was measured (in the model DU Beckman spectrophotometer) The two methods showed good agreement

The nucleotides formed during the phosphorylation of uridine in the presence of the rat liver enzymes were determined after the cooled incubation mixture was deproteinized with HClO₄ and neutralized to pH 7 with KOH The supernatant fluid was then chromatographed according to the procedure of Hurlbert *et al* (10) The individual nucleotides were identified by the coincidence of radioactivity with ultraviolet absorption in the presence of known carrier nucleotides, as well as by their migration on paper chromatograms in isobutyric acid-ammonia (15) and isopropanol hydrochloric acid (12) as compared with reference standards The extent of phosphorylation was usually followed by expressing radioactivity of the nucleotide forms as per cent of the total radioactivity recovered after incubation of uracil-2-C¹⁴ or uridine-2-C¹⁴ with the enzyme system in question In the experiments in which UMP was the only nucleotide formed or in which all the uridine nucleotides were determined collectively, the deproteinized and neutralized supernatant fluid was added to a 2 to 3 cm column of Dowex 1 resin in the formate form Elution commenced with two 5 ml portions of 0.2 N HCOOH, which elutes both uracil and uridine The

² Canellakis, E. S., *J. Biol. Chem.*, in press

column was further treated with two 5 ml portions of a mixture of 4.0 N HCOOH and 1.0 M HCOONH₄, which elutes the uracil nucleotides. Samples of the eluates were plated on stainless steel planchets (Tracerlab), were oven-dried for 10 to 16 hours, and were counted. This method volatilizes the ammonium formate. Control radioactive samples were similarly treated to permit evaluation of the self-absorption. The accuracy of the method was estimated to be within ± 8 per cent.

The PRPP was synthesized by the method of Kornberg *et al* (16), who employed an isoelectric precipitate of pigeon liver enzyme. After incubation for 2 hours, the mixture was cooled to 3° and 500 mg of activated charcoal were thoroughly suspended with 100 ml of incubation mixture and centrifuged. Of this supernatant fluid, 6 ml portions were added to 3 cm columns of activated charcoal and eluted with 5 ml of water. The two fractions obtained in this way, the break-through and the water eluate, contained, respectively, 75 and 25 per cent of the total PRPP, and 0.01 and 0.1 per cent of the total material absorbing at 260 m μ . Subsequent re-elution under similar conditions yielded PRPP, which was free from material absorbing at 260 m μ , it was assayed with the orotic acid phosphoribosyl-pyrophosphorylase enzyme obtained after alcohol fractionation of brewers' yeast (17).

Results

Uridine Phosphorylase—A partial purification of the enzyme, which resulted in a 10-fold increase in its specific activity (Table I), was carried out, at 0–3°, as follows. 1 gm of rat liver acetone powder was dissolved in 10 ml of 0.05 M Tris buffer, pH 7.4, and dialyzed for 24 hours against two 4 liter changes of this buffer. Under the conditions of preparation of the acetone powder, very little protein remained undissolved, this was centrifuged at 20,000 $\times g$ for 20 minutes. To the supernatant fluid (Fraction I), solid ammonium sulfate was added to 35 per cent saturation, a precipitate (Fraction II), obtained by centrifugation, contained about 75 per cent of the initial enzymatic activity, whereas the mother liquor (Fraction III) contained about 15 per cent of the initial enzymatic activity. Fraction II was dissolved in 10 ml of 0.05 M Tris buffer, pH 8.0, heated rapidly in a 65° water bath to 55° for 1 minute, and then quickly chilled and centrifuged. To the resulting supernatant fluid (Fraction IV) were added, per 5.0 ml, 1.25 ml of 1.0 M acetate buffer, pH 5.4, and 250 mg of calcium phosphate, after thorough mixing and centrifuging, the pH was adjusted to 7.4 with 1.0 M Tris buffer, pH 8.0 (Fraction V). The protein was precipitated completely by the addition of excess ammonium sulfate, dissolved in 0.05 M Tris buffer, pH 7.4, and dialyzed for 24 to 48 hours against three 4 liter changes of this buffer. The enzyme was assayed during the purification

procedure by the orcinol test which measures the R-1-P formed during the phosphorolysis of uridine

TABLE I
*Fractionation of Uridine Phosphorylase from Rat Liver Acetone Powders**

Fraction No	Total protein mg per sample†	Total enzyme activity per sample‡	Per cent of initial enzyme activity recovered	Specific activity§	Purification factor
I	20	106	100	5.3	1
II	6.4	79	74.5	12.3	2.3
III	9.6	15.9	15	1.66	0.3
IV	3.1	79	74.5	25.5	4.7
V	1.6	85	81	53.0	10.0

* Incubation medium contained 12.3 μ moles of uridine, 300 μ moles of phosphate, pH 8, 200 μ moles of Tris buffer, pH 8, and enzyme solution to a final volume of 3.7 ml. The volume of enzyme solution used in each tube was proportional to the total volume of the corresponding fraction.

† Protein determined by the method of Warburg and Christian (25) based on the equation given by Kalckar (26).

‡ Enzyme activity defined as the amount of ribose, in micrograms, formed in each sample during the first 5 minutes.

§ Ratio of total enzyme activity per sample to total protein mg per sample.

|| Mother liquor of Fraction II.

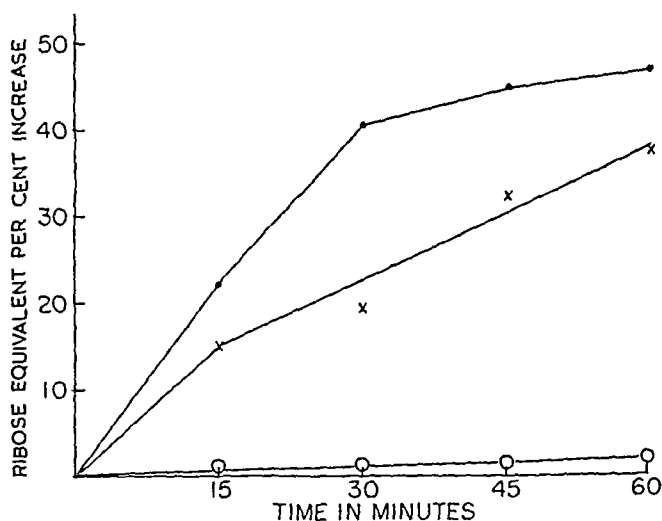


FIG. 1. Phosphorolysis of uridine by rat liver uridine phosphorylase. Incubation medium contained 12.3 μ moles of uridine, 200 μ moles of Tris buffer (pH 8), 22 mg of protein (Fraction I). O, no phosphate; X, 0.016 M phosphate; ●, 0.050 M phosphate. Values on abscissa measured as per cent of total uridine added.

Fig. 1 shows that uridine phosphorylase is inactive in the absence of phosphate and that its activity is dependent upon the concentration of

phosphate Of the end products of the reaction, the uracil was identified by paper chromatography (11), whereas the R-1-P was isolated as the barium salt by a slight modification of the method of Friedkin used for the isolation of deoxyribose 1-phosphate (18) Analysis of this barium salt for acid-labile phosphate and ribose showed a molar ratio of 1.00:1.06 In the reaction, as would be expected, arsenate was able to replace phosphate, while Mg^{++} was not required and EDTA did not inhibit Because of interfering reactions, it has been difficult to ascertain the equilibrium point of the reaction The requirement for R-1-P in the conversion of uracil to uridine was demonstrated by the decrease in the intensity of the orcinol-reactive material when uracil was incubated in the presence of an

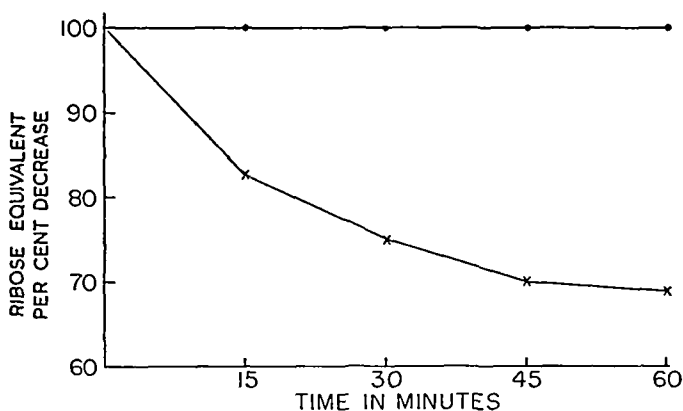


FIG. 2 Conversion of uracil and R-1-P to uridine in the presence of rat liver uridine phosphorylase. Incubation medium contained 15 μ moles of R-1-P, 200 μ moles of Tris buffer, pH 8, and 1.5 mg of protein (Fraction V), final volume, 4.0 ml. The reaction was followed by the disappearance of ribose color obtained with orcinol. ●, no uracil; X, 30 μ moles of uracil. Values on abscissa measured as per cent of initial ribose present.

excess of R-1-P (Fig. 2). This reaction did not occur when R-5-P or ribose was substituted for R-1-P.

The formation of uridine-2- C^{14} was demonstrated by paper chromatography when uracil-2- C^{14} was incubated with R-1-P in this system. Further evidence for the requirement of R-1-P for the conversion of uracil to uridine will be given when the properties of the uridine kinase are described. In Fig. 3, the pH-activity curve for the phosphorolysis of uridine is shown to have a rather wide optimum in the region of pH 8.

Assay of the crude acetone powder extract (Fraction I) for a cytidine nucleosidase or a cytidine phosphorylase showed these enzymes to be absent from this fraction, as assayed by the orcinol test.

Uridine Kinase—To the dialyzed and centrifuged acetone powder extract, described as Fraction I under "Uridine phosphorylase," was added

pyrophosphorylase, since we² have observed a similar inhibition of the orotic acid pyrophosphorylase from yeast (9) In this latter reaction, 0.15 M phosphate or 0.15 M sulfate also inhibited by 80 per cent, while 0.15 M chloride inhibited by 30 per cent

DISCUSSION

Previous investigations had shown that the soluble cytoplasmic fraction of rat liver contains the enzymes involved in the catabolism of uracil and of thymine (8) They had also indicated the requirements of the system as well as the nature of the compounds formed during this degradation The present investigation indicates that the same soluble cytoplasmic

TABLE III

*Phosphoribosylation of Uracil by Extracts of L. bulgaricus 09X in Presence of PRPP**

Additions†	Per cent conversion to UMP‡	
	Crude extracts	Fractionated extracts
Uracil-2-C ¹⁴ (0.05) + PRPP (0.05)	48	85
Uracil-2-C ¹⁴ (0.05) + R-5-P (0.20) + ATP (0.20)	12	2
Uracil-2-C ¹⁴ (0.05) + R-1-P (0.20) + ATP (0.20)		0
Uridine-2-C ¹⁴ (0.05) + ATP (0.20)		0

* Incubation medium contained 100 μ moles of Tris buffer, pH 8, 10 μ moles of Mg, 3.2 mg of protein for the crude extracts, and 1.1 mg of protein for the fractionated extracts Final volume, 1.0 ml, incubation time, 20 minutes

† The figures in parentheses define the micromoles of substrates added

‡ Per cent conversion of the total recovered radioactivity to UMP

fraction of rat liver contains the enzymes which participate in the anabolism of uracil The existence of enzymatic pathways in the liver which can convert uracil to the nucleotide stage contrasts with the very limited ability of this organ to incorporate uracil into RNA It is possible, of course, that the uridine phosphorylase and the uridine kinase under study normally have some other function, and that their action on uracil is incidental Another possibility, which is perhaps more likely, becomes apparent when the coexistence of the catabolic and anabolic pathways for some compound in the same cell fraction is taken into account This suggests a homeostatic mechanism which regulates the amount of compound available for anabolic reactions Such anabolic reactions, depending on the demands of the organism, could lead either to the production of precursors for nucleic acid synthesis or to the formation of precursors for the synthesis of uridine sugar

nucleotides, as for instance UDPG. Further evidence for the existence of such a homeostatic mechanism will be presented in a subsequent publication.² The occurrence of uridine phosphorylase has been previously demonstrated in *Escherichia coli* (24).

The demonstration of an enzyme in *L. bulgaricus* which will convert uracil to UMP in the presence of PRPP raises the possibility that this pathway for pyrimidine nucleotide anabolism may be found in other species. The sensitivity of this reaction to phosphate, sulfate, and chloride ions in combination with a variety of cations appears to be a reflection of a sensitivity of this reaction to high salt concentration rather than any particular anion or cation. This reaction affords a method for the enzymatic synthesis of UMP from uracil in good yields.

SUMMARY

The soluble cytoplasmic fraction of rat liver has been demonstrated to contain uridine phosphorylase, an enzyme which catalyzes the reversible reaction: uracil + ribose 1-phosphate = uridine + phosphate. This enzyme has been purified 10-fold. Cytidine is inert in this system.

The same fraction of rat liver contains uridine kinase, this enzyme, in the presence of adenosine triphosphate and Mg^{++} , phosphorylates uridine to form uridine 5'-phosphate. In addition, uridine di- and triphosphates and an unidentified phosphorylated uridine compound are formed.

The significance of these findings with reference to the very limited ability of rat liver to incorporate uracil into ribonucleic acid is discussed.

Some extracts of a uracil-requiring strain of *Lactobacillus bulgaricus* contain an enzyme which converts uracil to uridine 5'-phosphate in the presence of ribose 5-phosphate-1-pyrophosphate. This reaction affords a convenient method for the synthesis of radioactive uridine 5'-phosphate from radioactive uracil.

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ON THE MODE OF ACTION OF X-RAY PROTECTIVE AGENTS

III THE ENZYMATIC REDUCTION OF DISULFIDES*

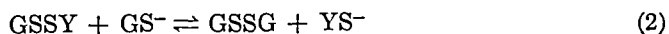
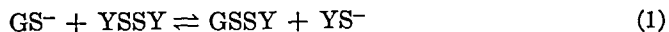
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It is well known that various disulfides can be reduced in animal tissues. Enzyme systems capable of reducing oxidized glutathione (GSSG) have been well characterized (1), but the detailed mechanism whereby other disulfides are reduced remains obscure. Recently Racker reported (2, 3) that homocystine was fairly rapidly reduced by a glutathione reductase system, provided small amounts of glutathione (GSH) were present. The reduction was thought to be due to a hydrogen transfer from GSH to homocystine, and this reaction was claimed to be catalyzed by a specific enzyme "glutathione-homocystine transhydrogenase" (3, 4). With crude liver preparations the reduction of a wide variety of SS compounds was observed, but in these instances no evidence for the participation of glutathione reductase or glutathione was obtained. No reduction of cystine could be demonstrated.

Recently it has been shown in this laboratory (5, 6) that the interaction of a thiol (*e g* reduced glutathione) with disulfides (YSSY) proceeds according to the following reactions



Clearly, if in such a thiol-disulfide system the GS^- is continuously regenerated by the action of glutathione reductase, the net result will be that YSSY is reduced. Since we have found that GS^- reacts rapidly with various disulfides at pH 7.4 and 37°, even at low concentrations of reactants, the possibility was considered (7, 8) that the biological reduction of disulfides may be brought about by Reactions 1 and 2, coupled with the enzymatic reduction of GSSG by glutathione reductase.

In the present report the role of glutathione in the mechanism of disulfide reduction has been reinvestigated. It is demonstrated that *N,N*-diacetylcystamine ((AcRS)₂), cystamine (RSSR), *N,N*-tetramethylcysta-

* Supported by grants from The Norwegian Cancer Society.

† Fellow of The Norwegian Cancer Society.

mine $((\text{Me}_2\text{RS})_2)$, N,N' -tetraethylcystamine $((\text{Et}_2\text{RS})_2)$, cystine (CSSC), and homocystine (HSSH) are reduced by a glutathione reductase preparation from rat liver in the presence of small amounts of glutathione. The data strongly support the mechanism proposed above, since in several mixtures of GS^- with disulfides the observed initial rate of disulfide reduction paralleled the equilibrium concentration of GSSG. The possibility that mixed disulfides containing glutathione (GSSY) could serve as substrates for glutathione reductase is excluded.

EXPERIMENTAL

Materials—Oxidized and reduced glutathione, triphosphopyridine nucleotide (TPN), and glucose 6-phosphate (the Ba salt) were purchased from the Nutritional Biochemicals Corporation. The glucose 6-phosphate was converted to the potassium salt. Tetramethylcystamine and tetraethylcystamine were kindly supplied by Deutsche Gold- und Silber-Scheideanstalt, Vormals Roessler, Frankfurt-on-the-Main. The L-cystine and DL-homocystine were obtained from Hoffmann-La Roche, Inc. Cysteamine, cystamine, and diacetylcystamine were synthesized according to procedures previously published (9, 6).

Preparation of Rat Liver Enzymes—An enzyme extract containing glucose-6-phosphate dehydrogenase and glutathione reductase was prepared from rat livers. The livers were squeezed through a tissue press, and the pulp thus obtained was homogenized in 2 volumes of 0.1 M phosphate buffer at pH 7.6. The homogenate was centrifuged at $100,000 \times g$ for 60 minutes. To the particle-free supernatant fluid 2 volumes of saturated ammonium sulfate were added. The protein was reprecipitated with ammonium sulfate, dissolved in phosphate buffer, and dialyzed against distilled water for 4 hours. 2 volumes of ice-cold acetone were added, and the precipitate was washed four times with acetone and subsequently lyophilized. The powder thus obtained could be stored in the cold for at least 2 months without loss of enzymatic activity.

Experimental Procedure—The reduction of GSSG and the other disulfides was measured according to the procedure of Rall and Lehninger (10). TPNH was prepared enzymatically from TPN and glucose 6-phosphate.

From Fig. 1 it is noted that the reoxidation of TPNH in the presence of disulfides, GSH, and glutathione reductase is very rapid compared to the reduction of TPN by glucose 6-phosphate. The loss of optical density at $340 \text{ m}\mu$ could therefore be used as a measure of the disulfide reduction rate.

Unless otherwise stated, each reaction mixture contained 0.5 ml of enzyme solution, 0.25 μmole of TPN, and 0.25 μmole of glucose 6-phosphate in 0.1 M potassium phosphate buffer at pH 7.6. The total reaction volume

was 2.15 to 2.20 ml. The TPN was reduced by incubating the reaction mixture for 1 hour at 25°, and the substrate solutions were then added.

The enzyme solution was obtained by extracting the acetone powder with 0.1 M phosphate buffer at pH 7.6 (20 mg of powder per ml of buffer). The faintly yellow solution was centrifuged ($25,000 \times g$ for 15 minutes) before use.

All substrate solutions were made 0.05 M with respect to Versene to prevent spontaneous oxidation of the sulfhydryl compounds. These Versene concentrations did not interfere with the reaction rates.

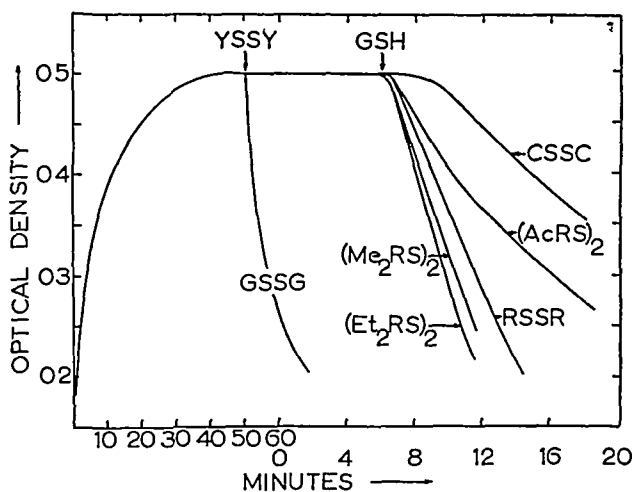


FIG. 1. The reduction of various disulfides by a rat liver glutathione reductase system. The reaction mixtures contained 0.5 ml of enzyme solution and 0.25 μ mole of TPN in 0.1 M potassium phosphate buffer at pH 7.6. At zero time 0.25 μ mole of glucose 6-phosphate was added. At the times indicated, 0.25 μ mole of the substrate (YSSY) and 0.5 μ mole of GSH were added. The incubation temperature was 25°. The optical density at 340 m μ was measured every 30 seconds.

Results

It appears from Fig. 1 that, except for GSSG, none of the disulfides tested was reduced by the enzyme preparation in the absence of GSH. When GSH was added, the different disulfides were reduced, although at markedly different rates. Also cystine was reduced under these conditions. These observations stand in contrast to the findings reported by Racker (3).

It is clear from the data in Fig. 1 that, whereas GSSG was readily reduced by the enzyme system, the other disulfides were unable as such to serve as substrates under our conditions. The fact that these disulfides were rapidly reduced upon addition of small amounts of GSH is most readily explained by assuming that the GSSG formed by the exchange

Reactions 1 and 2 was continuously reduced by the enzyme. The possibility that the mixed disulfides (GSSY) can likewise serve as substrates for the enzyme glutathione reductase should also be considered.

In order to test the above reaction mechanism we proceeded to measure the initial rate of reduction of various disulfides in different thiol-disulfide mixtures. The observed rates of reduction were correlated with the initial equilibrium concentrations of GSSG and GSSY in the substrate solutions. These latter concentrations were calculated by procedures previously reported (6), on the basis of the equilibrium constants K_2 and K_3 (Reactions 3 and 4) and the initial concentrations of the reactants.

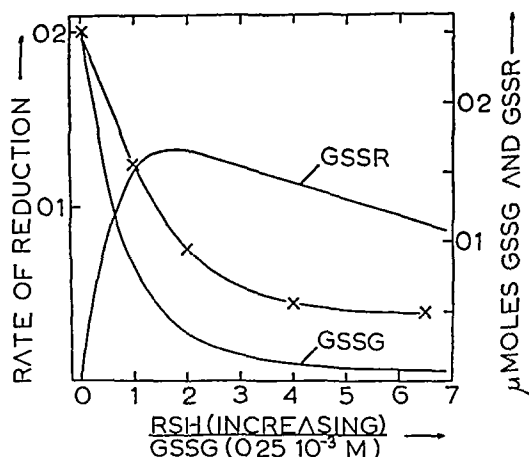


FIG. 2 The initial rate of disulfide reduction in reaction mixtures containing different equilibrium concentrations of the possible substrates (GSSG and GSSR). The substrate solutions were equilibrated for 10 minutes before the addition of the glutathione reductase system. The equilibrium concentrations of GSSG and GSSR were calculated (6) on the basis of $K_2 = 5.00$ and $K_3 = 0.34$ (unpublished data). Initial rate of reduction (X, $\Delta D_{340m\mu}$ per 30 seconds).

In Fig. 2 is shown the initial rate of disulfide reduction when a constant amount of GSSG was incubated, before the addition of the enzyme, with increasing amounts of cysteamine (RSH). It is clear that the initial rate of reduction paralleled the calculated equilibrium concentration of GSSG, while it showed no relationship to the GSSR concentration.

In mixtures of a constant amount of GSH and increasing amounts of $(AcRS)_2$ (Fig. 3), again a striking correlation between the calculated GSSG concentration and the initial rate of disulfide reduction can be seen. In Fig. 4 the same relationship is demonstrated in a second system (GSH + RSSR). Here is also included the rate of reduction in the GSH + HSSH system, the equilibrium constants of which are unknown. Clearly, the rate of reduction varies qualitatively as the expected variation of GSSG.

In both systems (Figs 3 and 4) the concentration of mixed disulfides showed no correlation with the rate of disulfide reduction

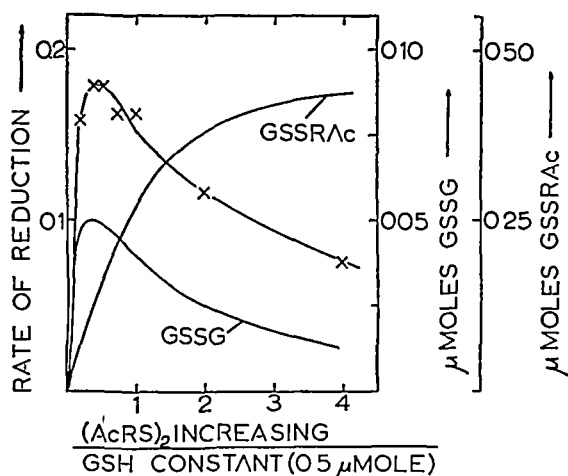


FIG 3 The initial rate of disulfide reduction in reaction mixtures containing different equilibrium concentrations of the possible substrates (GSSG and GSSRAc) Experimental conditions as in Fig 2 X, initial rate of reduction

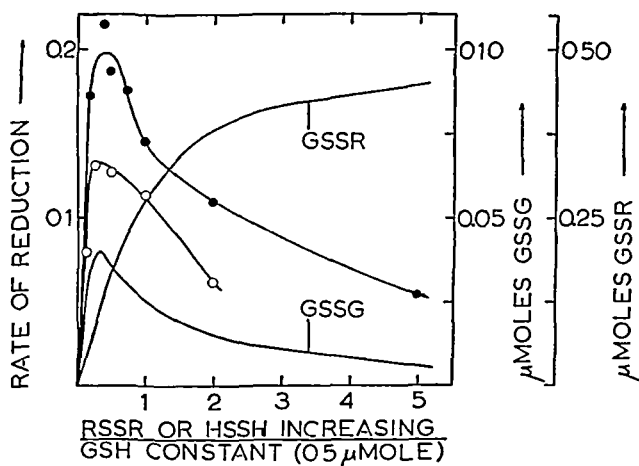
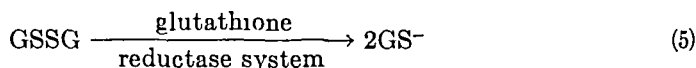
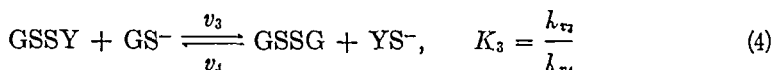
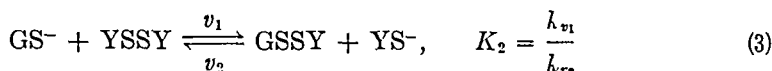


FIG 4 The initial rate of disulfide reduction in reaction mixtures containing different equilibrium concentrations of the possible substrates ●, initial reduction rate in the RSSR and GSH system The initial equilibrium concentrations of GSSR and GSSG refer to this system and are calculated on the basis of $K_2 = 2.94$ and $K_3 = 0.20$ (unpublished data) ○, initial reduction rate in the GSH and HSSH system The initial equilibrium concentrations of GSSG and GSSH are unknown

DISCUSSION

The present data demonstrate that a number of disulfides can be reduced by a glutathione reductase system in the presence of small amounts of glu-

tathione The finding that in several systems the initial rate of disulfide reduction parallels the initial equilibrium concentrations of GSSG in the substrate solution is considered evidence that these reductions are brought about by the following coupled reactions



The fact that the initial rate of disulfide reduction shows no correlation with the equilibrium concentration of GSSY rules out the possibility that mixed disulfides containing glutathione can serve as a substrate for glutathione reductase

It is observed in Figs 3 and 4 that, in the thiol-disulfide mixtures studied, the equilibrium concentrations of GSSG were very low. In fact, it can be calculated that the action of the glutathione reductase would rapidly have reduced the GSSG concentration virtually to zero, if GSSG were not promptly regenerated according to Reactions 3 and 4. The good agreement found between the initial rate of disulfide reduction and the equilibrium concentration of GSSG in the original substrate solution indicates that Reactions 3 and 4 were sufficiently fast to maintain the GSSG concentration. Under such conditions the equilibrium constants of the system determine the concentration of GSSG and thereby govern the over-all rate of disulfide reduction.

The reaction mechanism presented for the reduction of disulfides by glutathione reductase, in the presence of GSH, is generally applicable. So far, the mechanism has been adequately studied only in the two systems in which the equilibrium constants and the reaction rates had been determined. The studies are now being extended in this laboratory.

In the present studies no evidence was obtained for the participation of enzymes other than glutathione reductase. Although the possibility cannot be excluded that biological systems contain enzymes capable of catalyzing thiol-disulfide exchange reactions, the spontaneous reactions in the systems studied seem to be sufficiently rapid to make the postulation of such enzymes unnecessary.

The data in this paper support our previous contention (5, 6) that the accessible SH and SS groups in the organism exist in a dynamic equilibrium, governed by the appropriate equilibrium constants and by the ratio (total SH)/(total SS). This ratio presumably is regulated mainly by the

enzymes which oxidize and reduce glutathione. These views accord to glutathione a specific and important function in the regulation of the state of body SH and SS groups.

SUMMARY

The role of glutathione reductase in the mechanism of disulfide reduction has been reinvestigated. It is demonstrated that the disulfides, cystamine, diacetylcystamine, tetramethylcystamine, tetraethylcystamine, cystine, and homocystine, can be reduced by a rat liver glutathione reductase system, provided glutathione is present.

Under a variety of experimental conditions the initial rate of disulfide reduction was found to parallel the calculated initial concentration of oxidized glutathione in the reaction mixture, while it showed no relation to the concentration of the mixed disulfide. No evidence was found for the participation of other enzymes than glutathione reductase. On this basis it is concluded that the observed reduction of disulfides can be accounted for by a mechanism involving a two-step spontaneous thiol-disulfide exchange reaction (Reactions 3 and 4), coupled with the enzymatic reduction of oxidized glutathione (Reaction 5). It is also concluded that mixed disulfides containing glutathione cannot serve as substrates for glutathione reductase.

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ASSOCIATION OF SULFATE-S³⁵ WITH SERUM PROTEINS IN THE RAT

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After the intraperitoneal injection of sulfate-S³⁵ into rats, some of the isotope was shown, by electrophoresis in starch blocks, to be associated with the serum globulins as well as with the serum albumins (1). Smith *et al* have extended the observations on rat sera by the use of filter paper electrophoresis (2). Their results indicate that, 24 to 48 hours after intravenous injection of sulfate-S³⁵, about 40 per cent of the activity in the serum is associated with a serum constituent which has the electrophoretic mobility of an α_1 -globulin and which is stained intensely by fuchsin after pretreatment with periodic acid. They suggest that this S³⁵-labeled serum constituent may be a protein with a sulfated carbohydrate as its prosthetic group. It is possible, however, that some of the S³⁵ is present in the serum proteins as cystine and methionine, for, after the oral administration of sodium sulfate-S³⁵, cystine-S³⁵ and methionine-S³⁵ were isolated from the serum albumins of goats (3).

The experiments described here show that a small fraction of a dose of S³⁵, given as sulfate, can be recovered as cystine and methionine from the serum proteins of the rat. Most of the S³⁵ associated with the serum proteins of rats, however, is bound as sulfate, which can be released upon acid hydrolysis. Some of the bound S³⁵ can be extracted from the proteins as part of a non-dialyzable moiety. Evidence for the association of S³⁵ with the albumins and fibrinogen as well as with the α_1 -globulins is presented.

EXPERIMENTAL

Into each of twelve male rats of the Sherman strain, approximately 300 gm in body weight, 250 μ c of carrier-free S³⁵ as sodium sulfate in water were injected intraperitoneally.¹ The rats, having been anesthetized with ether, were then killed in groups of three after intervals of 6, 24, 48, and 72 hours. Blood was drawn directly from the heart and allowed to clot, and the serum was separated, pooled with like samples, and set aside at 0° until analyzed.

The total S³⁵ concentration in the sera was determined by counting the activity of the barium sulfate precipitates isolated on filter paper disks.

¹ The S³⁵ was obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.

after oxidation of 0.2 ml portions of the sera with Denis's reagent (4) as previously described (1). The S^{35} concentration was similarly determined in all the samples mentioned, unless otherwise indicated.

1 ml portions of the sera were dialyzed against 25 ml portions of water in rocking dialyzers (5) for 24 hours at 0° , and the S^{35} in the dialysates and residues was determined.

To 1 ml portions of the sera, 9 ml of a cold 5 per cent solution of trichloroacetic acid were added with stirring. The tubes were set aside at 0° for 30 minutes and then centrifuged. The precipitates were resuspended in fresh 5 ml portions of the 5 per cent solution of trichloroacetic acid and recentrifuged, this procedure was repeated. The washes were combined with the initial supernatant solution, and the whole was evaporated to dryness on a steam bath. S^{35} was determined in the resultant residue and in the proteins precipitated by the trichloroacetic acid.

3 ml of 95 per cent ethanol were added to 1 ml portions of the pooled sera. The precipitates were isolated 1 hour later by centrifugation and were then extracted two times with 3 ml portions of 75 per cent ethanol in the centrifuge. The extracts were added to the supernatant solution obtained when the proteins were initially precipitated, and the solution was evaporated to dryness on a steam bath. S^{35} was determined in the resultant residue and in the proteins precipitated by the ethanol.

A 2 ml portion of serum from each pool was subjected to electrophoresis in a starch block (6) for 6.5 hours at 600 volts. Barbitol buffer, pH 8.6, 0.1 μ , was used with starch blocks approximately 1 cm thick, 5 cm wide, and 62 cm long. Under these conditions inorganic sulfate was separated from the proteins but was still present on the starch block (1). The starch was cut into segments approximately 1 cm wide, each of which was extracted with 2 ml of a 1 per cent solution of sodium chloride. After removal of the starch by filtration through a sintered glass funnel, a 0.1 ml aliquot of the filtrate was taken for the estimation of protein by a modified tyrosine method (7). The S^{35} in a 1 ml portion of each filtrate was determined by delivery into a stainless steel planchet, by drying under an infrared lamp, and then by counting with a thin window Geiger-Müller tube.

Each of four adult male rats, weighing 300 to 350 gm, received by intraperitoneal injection 500 μ c of carrier-free S^{35} as sodium sulfate in water. The rats were killed in groups of two, 24 and 48 hours later. Approximately one-half of each blood sample, when drawn, was delivered into a tube containing oxalate, and the remainder was allowed to clot. Samples of plasma and serum from the rats killed at the same time were separately pooled, and 2 ml portions from each pool were dialyzed in rocking dialyzers against 25 ml portions of water at 0° for 24 hours. Electrophoresis of the

proteins in a starch block with barbital buffer of pH 8.6, 0.1 μ , followed Starch blocks 22 cm wide, 45 cm long, and approximately 1 cm thick were used, so that on one-half of the block the serum proteins and on the other half the plasma proteins from the same rats could be separated simultaneously. Separation was allowed to proceed for 48 hours at 0° and an applied potential difference of 300 volts. Each half of the starch block was cut into segments 1 cm wide and each of these was then extracted with two portions of 5 ml of a 1 per cent solution of sodium chloride. The protein was determined in 0.5 ml portions of the filtrates (7), and the S^{35} as follows. A 5 ml portion of the filtrate was delivered into a 600 ml Pyrex beaker which contained 5 ml of a 0.05 N solution of sodium sulfate and 10 ml of an 8 N solution of hydrochloric acid. The resultant solutions were evaporated to dryness on a steam bath, the residues were dissolved in 250 ml of water, and the sulfate was precipitated by the addition of 5 ml of a 10 per cent solution of barium chloride. The volume was reduced to about 75 ml by boiling, and the barium sulfate was isolated for counting 24 hours later by filtration on to filter paper disks.

Each of six adult rats, three males and three females, received intraperitoneally 1250 μ c of carrier-free S^{35} as sodium sulfate in water 24 hours before being killed. Approximately 3 ml of each blood sample were delivered into a tube containing oxalate, and the remainder was allowed to clot. The S^{35} was determined in 0.2 ml portions of the whole blood. After centrifugation for 30 minutes at 3000 r.p.m., the packed cell volume was noted. Plasma or serum was separated from the cells or clot, respectively. S^{35} was determined in the serum and plasma.

The proteins in 0.01 ml portions of the plasmas and sera were separated by electrophoresis on filter paper. Sheets of Whatman 3 MM paper, 26 cm wide and 54 cm long, were used with barbital buffer of pH 8.6, 0.1 μ . The applied potential difference was 300 volts at 0° for 24 hours. After drying the paper in air, it was placed in contact with x-ray film for 2 weeks. The x-ray film underwent routine development, and the paper was stained with tetrabromophenolsulfonphthalein (7).

From the pooled sera, each of twelve 0.4 ml portions was dialyzed against 25 ml of water at 0° for 4 hours in a rocking dialyzer (5). After the water was changed, dialysis was continued for 14 hours longer. At this time, in four of the dialyzers 25 ml of 1 N hydrochloric acid solution replaced the water and 1 ml of the acid solution was also added to the protein solution. In four other dialysis units, 1 N solution of sodium hydroxide similarly replaced the water. In the remaining four units, the change was to fresh water. At the end of 4 hours, the acid, the alkali, and the water were replaced by fresh solutions, and dialysis was continued for an additional

20 hours The S³⁵ in the dialysates and residues was determined after acid hydrolysis

To 2 ml of the pooled sera 3 ml of water were added and then, slowly with stirring, 5 ml of a 10 per cent solution of trichloroacetic acid 30 minutes later the precipitated proteins were separated by centrifugation and washed three times with 10 ml portions of a 5 per cent solution of trichloroacetic acid The proteins were then hydrolyzed by boiling under a reflux for 6 hours with 25 ml of 6 N hydrochloric acid The hydrolysate was decolorized with Darco G-60 charcoal and evaporated to dryness The residue was dissolved in 3 ml of water A 2 ml portion of the solution was placed on a 0.9 by 52 cm long column of Dowex 50-X8 (H⁺), 200 to 400 mesh Elution with water and hydrochloric acid followed (8) The effluent solution was collected in 2 ml fractions, of which 1 ml was delivered into a glass planchet After drying under an infrared lamp the S³⁵ was determined with a thin window Geiger-Muller tube

A 4 ml portion of the pooled sera was diluted with 21 ml of water and electrodialyzed at 0° Cellophane membranes and platinum foil electrodes were used After 24 hours, electrodialysis was stopped, and the water in the end chambers was replaced by 500 ml portions of 0.1 M phosphate buffer of pH 7.5, and to the middle compartment 25 ml of 0.2 M phosphate buffer of the same pH, containing 12.5 mg of trypsin, were added Incubation for 36 hours at 25–30° followed, while the buffer in the end compartments was being stirred with air To the solution from the middle compartment, 50 ml of a 10 per cent solution of trichloroacetic acid were added slowly with stirring 30 minutes later insoluble material was removed by centrifugation and washed twice in the centrifuge with 100 ml portions of a 5 per cent solution of trichloroacetic acid The combined supernatant fluids were diluted to 400 ml with water S³⁵ was determined in 10 ml aliquots after acid hydrolysis The remainder of the solution was electrodialyzed and then evaporated to dryness in a flash evaporator The residue was dissolved in 10 ml of water, and the concentrations of S³⁵, glucuronic acid (9), and hexosamines (10) therein were determined

The protein precipitated by trichloroacetic acid after treatment with trypsin was dissolved in 50 ml of a 2 per cent solution of sodium hydroxide and the S³⁵ was determined with 5 ml aliquots

Two adult rats each received 1500 μ c of carrier-free S³⁵ as sodium sulfate in water They were killed 20 hours later Blood was drawn into tubes containing oxalate After the plasma was separated from the cells, the buffy layer of cells was transferred to a Shevsky-Stafford tube containing 5 ml of a 1 per cent solution of sodium chloride After centrifugation, the saline solution was discarded and the cells were transferred again to a Shevsky-Stafford tube which contained 5 ml of the saline solution Centrifugation and washing were repeated four more times Finally the total

volume of cells and the volume of white cells were noted. The concentration of S^{35} in the sample of cells was determined. Samples of red cells were similarly washed and analyzed for S^{35} .

RESULTS AND DISCUSSION

From the data presented in Fig 1, it can be seen that, as the concentration of S^{35} , given as sodium sulfate, decreases precipitously in the rat serum,

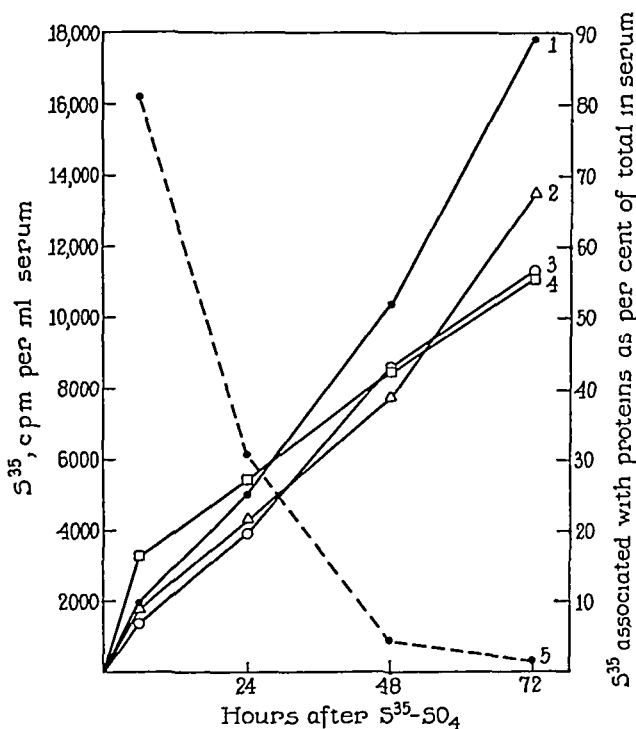


FIG 1 The fraction of total S^{35} associated with proteins of rat serum after intervals of time subsequent to the intraperitoneal injection of sodium sulfate- S^{35} . The results were obtained (1) with electrophoresis in a starch block for 6.5 hours at an applied potential difference of 600 volts, (2) with equilibrium dialysis against water, (3) by precipitation of the proteins with 5 per cent trichloroacetic acid, (4) by precipitation of the proteins at an ethanol concentration of 75 per cent. The change in the concentration of S^{35} in rat serum with time is shown in Curve 5. The sera were from adult rats, each of which had received $250 \mu c$ of sulfate- S^{35} .

an increasing fraction of the remaining isotope is associated with the serum proteins. Approximately similar values for this fraction are obtained whether one uses electrophoresis in starch blocks, dialysis against water, precipitation of the proteins by trichloroacetic acid, or precipitation by ethanol.

In order to ascertain whether the S^{35} was associated with one or more of the protein components in the serum and plasma of rats, the protein com-

ponents were separated by extended electrophoresis on starch blocks and on filter paper. A typical electrophoretic pattern of the serum proteins as separated in a starch block is shown in Fig 2, *b*. By comparing this pattern with the pattern of S³⁵ concentrations in the starch block (Fig 2, *a*), it is seen that most of the isotope is associated with the protein or proteins which move as does α_1 -globulin. This observation is in accord with the findings of Smith *et al* (2), based on electrophoresis of rat sera on filter paper. A significant amount of the S³⁵ is, however, also associated

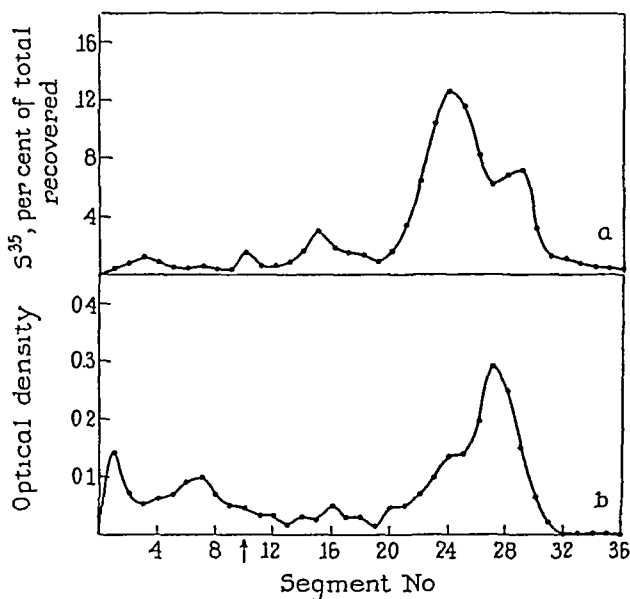


FIG 2 Electrophoretic patterns of adult rat serum 48 hours after the intraperitoneal injection of sodium sulfate-S³⁵. The separation was effected in barbital buffer, pH 8.6, 0.1 μ , in a starch block at an applied potential difference of 300 volts for 48 hours. The S³⁵ (*a*) and protein (*b*) contents in 1 cm wide segments were determined as detailed in the text.

with the albumin fraction, and small amounts with the globulins other than the α_1 -globulin. With the exception that slightly more activity was found in the region of the γ -globulins when plasma was used, the electrophoretic analyses of plasma gave results similar to those obtained with serum.

The electrophoretic separation of the proteins of rat sera and plasmas on filter paper is shown in Fig 3, *A*, and the autoradiograms of the paper before staining in Fig 3, *B*. Here again it is seen that S³⁵ is associated mostly with the albumins and the α_1 -globulins. A less intense reaction is also given by regions of the paper to which the other globulins have migrated. Of particular interest is the indication on the autoradiograms that in the region occupied by the γ -globulins there is a rather irregular and

stronger reaction when plasma is used instead of serum. This may be due to the presence in rat fibrinogen, as in bovine fibrinogen, of tyrosinyl-O-sulfate radicals (11).

Having established that S^{35} associates with the serum and plasma proteins of rats after the administration of S^{35} -labeled sulfate, it was of interest to determine whether any of the S^{35} was present in the proteins as cystine and methionine. Chromatography of a hydrolysate of serum proteins on Dowex 50 showed that, of the total S^{35} associated with the proteins

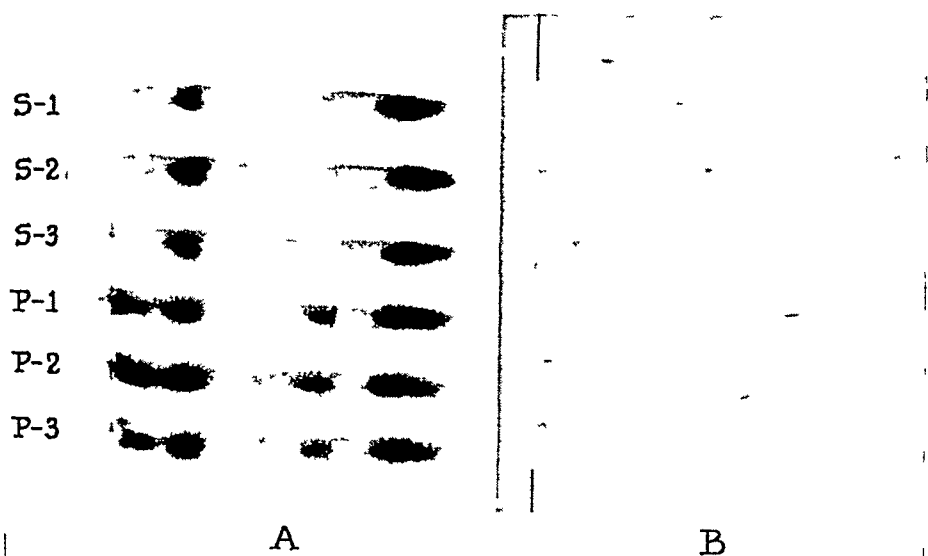


FIG. 3. Electrophoretic patterns of serum (S) and plasma (P) proteins on filter paper, A, and the autoradiograms of the protein patterns produced on X-ray film, B, before the paper was stained with tetrabromophenolsulfonphthalein. The sera and plasmas were from rats that had received intraperitoneally 1250 μ C of S^{35} as sodium sulfate 24 hours previously. It can be seen that S^{35} was present in the positions to which the albumins and α_1 -globulins had migrated. In the positions occupied by the other globulins, small amounts of S^{35} were also present. $\times 0.267$.

24 hours after injection of the S^{35} -labeled sulfate, 5.1 per cent was present as cystine and methionine sulfur. The remainder of the S^{35} was eluted from the column of resin as inorganic sulfate. These observations are in accord with reports (12-14) that only an exceedingly small, sometimes undetectable, fraction of a dose of sulfate-sulfur can be recovered from rat tissues as cystine and methionine sulfur.

Carbohydrate-protein complexes have been isolated from human plasma (15-17), and the suggestion that they contain labile sulfate groups (15, 16) is substantiated in part by the recent isolation of chondroitin sulfate from normal human plasma (18). Therefore, it was not surprising that, after

the elimination of most of the proteins from 4 ml of rat serum by tryptic digestion, precipitation with trichloroacetic acid, and electrodialysis, 30.6

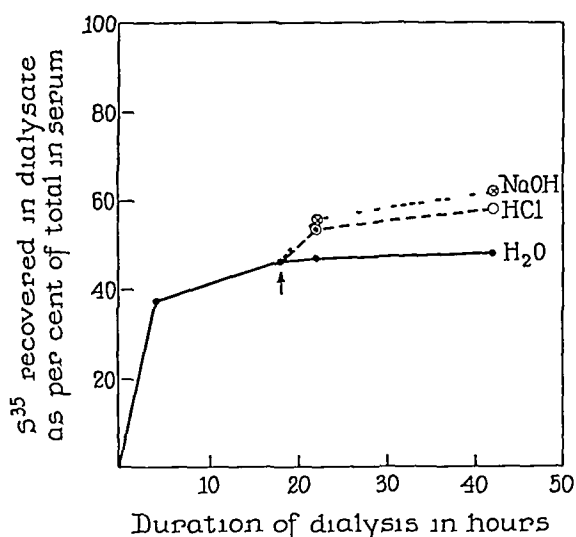


FIG. 4. Effect of the substitution of 1 N hydrochloric acid and 1 N sodium hydroxide solutions for water on the recovery of S³⁵ in dialysates of rat serum. From a pool of sera, obtained from rats 24 hours after each animal received intraperitoneally 1250 μ c of S³⁵ as sodium sulfate, 0.4 ml samples were dialyzed against 25 ml portions of water at 0°. The water was changed periodically. After 18 hours some of the samples were dialyzed against acid or alkali at 0°. Each point is the average value on four samples.

TABLE I

S³⁵ in Blood, Plasma, and Serum of Adult Rats 24 Hours after Intraperitoneal Injection of Sulfate-S³⁵

Each rat received 1250 μ c of carrier-free S³⁵ as sodium sulfate in water

Rat No	Body weight	Blood	Plasma	Serum	Cells*
		c p m per ml	c p m per ml	c p m per ml	c p m per ml
1	270	11,220	29,460	20,625	2,980
2	281	11,100	18,465	19,095	3,736
3	310	12,685	20,855	20,420	4,516
4	250	16,445	30,240	29,525	2,650
5	228	24,560	41,605	44,000	7,516
6	222	27,550	37,520	41,605	17,580

* The packed cell volume was found to represent 47 to 52 per cent of the total blood volume. The concentration of S³⁵ in the cells was calculated with 50 per cent as an average value for the packed cell volume ((S³⁵ in 1 ml blood) - $\frac{1}{2}$ (S³⁵ in 1 ml plasma)) \times 2 = S³⁵ in 1 ml cells.

per cent of the S³⁵ originally associated with the proteins was recovered in solution with 80 γ of glucuronic acid and 1940 γ of hexosamine.

Apparently the sulfate which goes along with the serum proteins is strongly bound. It is not removed by dialysis against changes of water at 0° for up to 42 hours, or by electrophoresis in starch blocks at pH 8.6 and 0° for 48 hours when the applied potential difference is 300 volts. Values for bound S^{35} similar to those obtained by the two techniques just mentioned were obtained also when the proteins were precipitated by a concentration of 75 per cent ethanol or 5 per cent trichloroacetic acid (Fig. 1). It was found, however, that at 0°, when sera were dialyzed against 1 N hydrochloric acid solutions or 1 N sodium hydroxide solutions, more sulfate could be recovered in the dialysates than when dialysis was against water (Fig. 4). The additional amounts of sulfate thus recovered were approximately the same whether sera were dialyzed against the acidic or alkaline solutions.

TABLE II

*S^{35} in Blood, Plasma, Red Cells, and White Cells of Adult Male Rats
20 Hours after Intraperitoneal Injection of Sulfate- S^{35}*

Each rat received 1500 μ c of carrier-free S^{35} as sodium sulfate in water

Rat No	Body weight	Blood	Plasma	Red cells	White cells*
	gm	c p m per ml	c p m per ml	c p m per ml	c p m per ml
7	263	114,850	174,000	50,800	66,700
8	345	69,500	91,750	25,300	53,800

* Calculated after subtraction of the activity associated with the red cells which were present in the sample analyzed

From the data in Table I it is evident that the concentration of S^{35} in the plasma does not differ significantly from that in the serum. Calculations based on the concentration of S^{35} in whole blood and in plasma suggest that a significant amount of the isotope was present also in the cells. The white cells apparently take up more of the S^{35} in 20 hours than do the red cells (Table II) as reported also by Odell *et al* (19).

SUMMARY

After the intraperitoneal injection of sodium sulfate- S^{35} into rats, as the concentration of S^{35} in the sera decreased an increasing fraction of the remaining isotope was associated with the serum proteins.

Electrophoretic analysis of sera showed that most of the S^{35} was associated with the α_1 -globulins and the albumins. When plasma was used, it was also seen that S^{35} was associated with fibrinogen.

Chromatography on Dowex 50 resin of hydrolysates of proteins from sera, removed 24 hours after injection of sulfate- S^{35} , revealed that about 5 per

cent of the isotope associated with the proteins was present as cystine and methionine and the remainder as sulfate

After tryptic digestion and precipitation of undigested proteins with trichloroacetic acid, about 30 per cent of the S³⁵ originally associated with the serum proteins was recovered as part of a non-dialyzable component. Glucuronic acid and hexosamine were also present in this solution

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A MICROMODIFICATION OF THE SMITH AND ROE METHOD FOR THE DETERMINATION OF AMYLASE IN BODY FLUIDS

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The procedure developed by Smith and Roe (1) for the determination of amylase in body fluids, in which the starch-iodine color reaction is utilized, is limited for clinical and experimental purposes by the size of the sample required, which is 2 ml. This procedure also uses a large volume of water, 500 ml, in the color development, which is inconvenient and not economical. In the following micromodification, the quantity of the sample analyzed is reduced to 0.2 ml, and the volume of water used for diluting the color is decreased to 200 ml. The method is greatly simplified and the speed and accuracy of the original procedure are retained.

Reagents—1 Phosphate buffer-sodium chloride solution. A 0.06 M phosphate buffer solution, pH 7.2, which is 0.05 M in sodium chloride, is prepared as follows: 2.922 gm of sodium chloride, 6.135 gm of anhydrous disodium hydrogen phosphate, and 2.286 gm of anhydrous potassium dihydrogen phosphate are dissolved in distilled water in a liter flask and the volume is made up to 1 liter.

2 Substrate solution. A 0.3 per cent suspension of soluble starch in the phosphate buffer-sodium chloride reagent is made up at the time of use. 300 mg of Lintner soluble starch (Merck) are weighed accurately and suspended in about 10 ml of cold phosphate buffer-sodium chloride solution in a 100 ml volumetric flask, which is agitated until all of the starch is brought into a homogeneous suspension. The suspension is made up to volume with boiling phosphate buffer-sodium chloride reagent and mixed, and the flask is placed in a boiling water bath for 3 minutes. The starch mixture is transferred to a 125 ml Erlenmeyer flask to facilitate mixing and pipetting, and this flask is placed in the water bath and allowed to cool to about 90°. The starch suspension is maintained at constant temperature during pipetting.

Starch solutions prepared in this manner will keep in the refrigerator for a period of 2 weeks with no greater than a 10 per cent loss in the susceptibility of the substrate to hydrolysis by amylase. When using the refrigerated substrate, mix thoroughly and pour slightly more than will be used into a large test tube. Place the tube in a boiling water bath for 3 minutes,

allow the bath and substrate to cool to 90°, and maintain at a constant temperature, as before, while pipetting

3 N hydrochloric acid

4 Iodine reagent 18 gm of potassium iodide and 1.8 gm of iodine are dissolved in distilled water in a liter flask and the solution is made up to volume

Procedure

2 ml of the substrate solution are pipetted into each of two 15 × 125 mm rimmed test tubes and placed in a water bath at 37° until temperature equilibration is reached. 0.1 ml of whole blood, serum, plasma, or urine is added to one tube. The second tube is retained as a control, and both tubes are incubated for one-half hour. At the end of the incubation period, the contents of the tubes are poured into labeled 200 ml volumetric flasks containing about 100 ml of distilled water and 3 ml of N HCl. The tubes are rinsed four times with distilled water and the washings are added to the flasks. 0.1 ml of the sample being analyzed (whole blood, serum, plasma, or urine) is added to the control flask and 1 ml of iodine reagent is added to each flask. The flasks are made up to volume and mixed thoroughly. After 15 minutes, the colored solutions are compared in a photoelectric colorimeter at 620 mμ, distilled water being used to set the instrument at zero absorbance. The control flask gives the starch-iodine color value without amylase action and the digest flask gives the value remaining after amylase action.

Calculations—If D = optical density, then $((D \text{ of control}) - (D \text{ of digest})/D \text{ of control}) \times 6 = \text{mg of starch hydrolyzed}$. The amylase unit is defined as the amount of enzyme that, under the conditions of this procedure, will hydrolyze 10 mg of starch in 30 minutes to a stage at which no color is given with iodine.

For whole blood, plasma, serum, or urine, the calculation then becomes $((D \text{ of control}) - (D \text{ of digest})/D \text{ of control}) \times 6/10 \times 100/0.1 = \text{amylase units per 100 ml}$.

When High Values Are Obtained—If the experimental tube gives an optical density between 0.1 and 0.02 (80 to 95 per cent transmittance), dilute the sample being analyzed 1:1 with saline, for optical densities less than 0.02 (above 95 per cent transmittance), dilute 1:4 with saline and repeat the test.

Comparison with Original and Somogyi Methods—Serum amylase values were determined on fifteen fasted human subjects by the proposed micro-method and by the original Smith and Roe procedure. The values (Table I) are averages for three determinations. It was found that the micro-procedure gave higher results than the macromethod, by a ratio of 1.21:1.0.

In the original report (1), comparative analyses upon normal subjects, with the method published and the Somogyi method (2), showed that the values obtained by the Somogyi method are 10 per cent higher than those obtained by Smith and Roe. Since the micromodification, as shown in Table I, gave results that are 21 per cent higher than those obtained by the macro method, it follows that the proposed microprocedure gives values approximately 10 per cent higher than would be obtained by the Somogyi method. For practical purposes, the results by the proposed micro-

TABLE I
*Comparison of Serum Amylase Levels by Smith-Roe Procedure and
by Proposed Micromodification*

Subject No	Original method	Proposed micromodification	<u>Micro</u> <u>Macro</u>
	<i>units per 100 ml</i>	<i>units per 100 ml</i>	
1	38	46	1 21
2	48	57	1 19
3	58	73	1 25
4	53	60	1 13
5	75	86	1 15
6	58	67	1 15
7	59	65	1 10
8	46	58	1 26
9	69	85	1 23
10	59	74	1 25
11	81	96	1 19
12	44	55	1 25
13	56	71	1 27
14	50	63	1 26
15	60	74	1 23
Mean	57	69	1 21

procedure may be considered as essentially comparable to the data obtained by the Somogyi method (2).

The variation in results obtained by the micro- and the macroprocedures was found to be due to a variation in the method of preparation of the starch substrate. The concentration at which the starch was originally prepared was the determining factor in the amylase activity of a given serum sample. Table II shows the amylase values by the proposed method obtained with starch solutions prepared in concentrations of 1.2 and 0.3 per cent and in varying final dilutions. The amount of starch present was the same (6 mg) in each digestion mixture. The results show that dilution of a previously prepared substrate, made at the time of the analysis, had

no effect, but the concentration at which the starch was originally prepared had a marked effect. The starch heated in a suspension of lower concentration was considerably more susceptible to amylase action.

Substrate Studies—In view of the great advantage to the clinical laboratory of having a substrate that may be kept in the refrigerator for considerable periods of time and used as needed, studies were carried out on the keeping qualities of a number of starch preparations. An entirely satisfactory preparation was not found. The preparations tested included the starch-phosphate buffer-NaCl solution reported herein, the same solution containing 10 per cent glycerol, the same solution containing 10 per cent glycerol and saturated with thymol as a preservative, and a solution in which phosphate buffer was replaced by a tris(hydroxymethyl)amino-

TABLE II
Serum Amylase Values at Varying Substrate Concentrations

Substrate		Final volume	Determination No			Average
			1	2	3	
	ml	ml	units per 100 ml serum	units per 100 ml serum	units per 100 ml serum	units per 100 ml serum
1 2% starch	0.5	1	77	74	77	76
1 2% "	0.5	2	77	77	80	78
0.3% "	2.0	2	91	91	91	91
0.3% "	2.0	4	91	92	94	92

methane (Tris) buffer, pH 7.2, prepared according to Gomori (3). Substrates were prepared and kept in the refrigerator until time for use.

The results shown in Table III are typical of all the solutions tested. Some precipitation of starch occurred in all solutions in 2 weeks, and extensive precipitation with considerable loss in susceptibility to enzyme action took place in 3 weeks. The decreased susceptibility to enzyme action during the first 24 hours was not accompanied by any apparent changes in the appearance of the solution, but there was a slight decrease in the capacity of the starch to produce a blue color with iodine. This decrease in the intensity of color produced with iodine became progressively greater with time.

In these studies Tris buffer gave results identical to those obtained with phosphate buffer, within experimental error. The former reagent can, therefore, serve as a substitute for the phosphate buffer in the determination of amylase, if it is desired to limit the concentration of phosphate.

An attempt was made to remove the material precipitated from the substrate mixtures by filtration previous to refrigeration. Six different grades

of filter paper and three different porosities of sintered glass filters were used. Filtration removed some of the starch, as evidenced by the de-

TABLE III

Studies on Keeping Qualities of Starch Preparations

Action of urinary amylase on the proposed substrate kept in the refrigerator for varying periods of time. Human urine, diluted 1:4 with distilled water, served as the source of enzyme.

Time in refrigerator	Units per 100 ml. when pipetted		Precipitation of substrate
	Hot	Cold	
<i>hrs</i>			
0	150		None
24	138	129	"
<i>wks</i>			
1	136	127	"
2	135	122	Some
3	110	98	Extensive

TABLE IV

Distribution of Serum Amylase Values of 122 Fasted and 62 Non-Fasted Subjects

Amylase value	No. of fasted subjects	No. of non fasted subjects
<i>units per 100 ml</i>		
10-20	1	1
20-30	6	1
30-40	9	0
40-50	27	3
50-60	14	8
60-70	23	17
70-80	22	12
80-90	8	5
90-100	1	7
100-110	5	4
110-120	4	2
120-130	1	0
130-140	1	2
Total	122	62

creased intensity of color produced with iodine, but had no effect on the subsequent precipitation of the substrate or its susceptibility to enzyme action.

Normal Serum Amylase Value—Before the micromodification reported in this paper was developed, analyses were made with the regular proce-

ture (1) designed to establish the range of normal serum amylase values of human subjects. The individuals tested were medical and graduate students between the ages of 18 and 36 years, who were apparently in good health. The groups included 122 subjects who were fasted overnight and 62 subjects who were not fasted. The results are shown in Table IV.

The serum amylase values of the fasted group ranged between 19.8 and 134 units per 100 ml, with a mean of 62.2 units, the standard deviation was ± 22.99 and the standard error of the mean was ± 2.07 . The range of values for the non-fasted subjects was 18.9 to 136.6 units per 100 ml. The mean value of this group was 74.3 units, with a standard deviation of ± 22.39 and a standard error of the mean of ± 2.84 . Statistical analysis of these data showed a significant difference ($P < 0.01$) between the fasted and the non-fasted groups.

The highest values observed in the fasted and non-fasted groups were 134 and 137 units per 100 ml, respectively. If the micromodification had been used, the highest normal values would have been 162 and 165 units per 100 ml for the fasted and non-fasted groups, respectively, calculated with the conversion factor of 1.21 mentioned above.

SUMMARY

1. A micromodification of the Smith and Roe method for the determination of amylase in body fluids has been developed. The method has been simplified and the speed and accuracy of the original procedure have been retained.

2. This procedure gives values 21 per cent higher than the original method. The differences in the results obtained by the two methods are due to a variation in the concentration of starch used at the time of preparation of the substrate. Dilution of the starch suspension after the substrate had been prepared had no effect on the activity of the enzyme.

3. The range of serum amylase values of normal human subjects by the original Smith and Roe procedure, per 100 ml, was found to be 19.8 to 134 units for 122 fasted subjects and 18.9 to 136.6 units for 62 non-fasted individuals.

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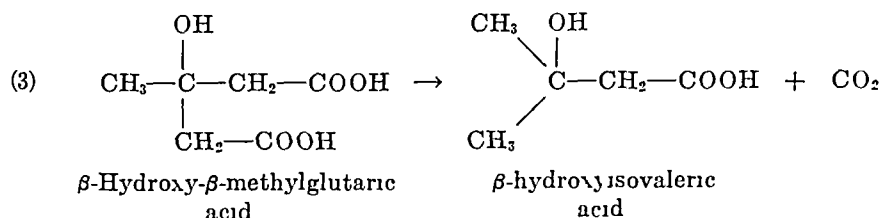
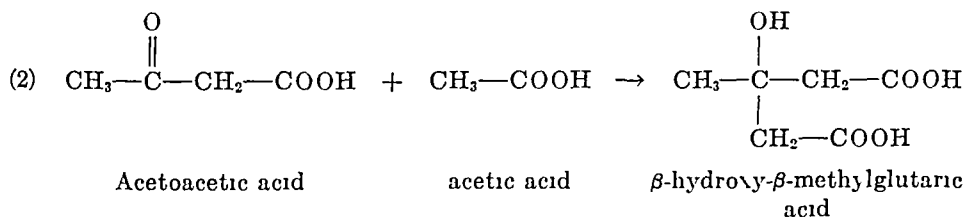
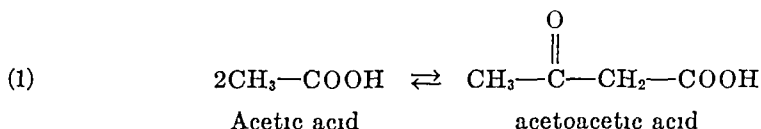
THE BIOSYNTHESIS OF β -HYDROXY- β -METHYLGLUTARIC ACID*

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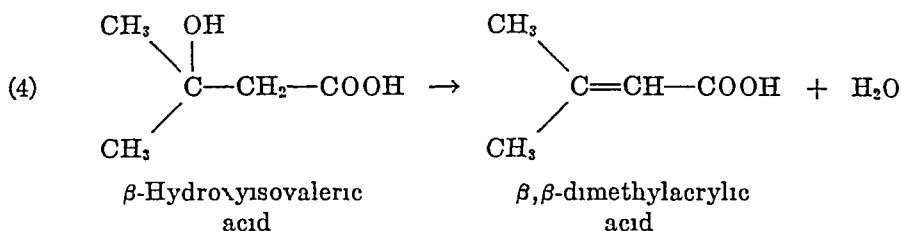
Previous work has shown that animal and plant tissues are able to synthesize branched chain fatty acids such as β -hydroxy- β -methylglutaric acid,¹ β -hydroxyisovaleric acid, and β , β -dimethylacrylic acid from smaller precursors such as acetic acid (1-7). This synthetic pathway appears to involve the following reactions wherein it is assumed that all the acids are activated as acyl CoA derivatives



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† Scholar in Cancer Research of the American Cancer Society.

¹ The following abbreviations are used: β -hydroxy- β -methylglutaric acid, HMG, coenzyme A, CoA, reduced coenzyme A, CoA SH, acetyl coenzyme A, Ac CoA, acetoacetyl coenzyme A, AcAc CoA, adenosine triphosphate, ATP, β -hydroxy- β -methylglutaryl coenzyme A, HMG CoA.



The distribution of C^{14} in HMG, β -hydroxyisovaleric, and β,β -dimethylacrylic acids observed when $\text{C}^{14}\text{H}_3\text{COOH}$ was incubated with cell-free rat liver preparations is in agreement with the foregoing sequence of reactions. These activated branched chain fatty acids or closely related derivatives thereof appear to be precursors in the synthesis of cholesterol, since the pattern of labeling found in the branched chain fatty acids closely resembles that found in squalene (8) and cholesterol (9, 10). Reactions 2, 3, and 4 have been studied in the reverse direction by Bachhawat *et al* (11). These workers found that the formation of acetoacetate and acetyl CoA from dimethylacrylyl CoA involved an obligatory hydration with crotonase followed by an ATP-requiring CO_2 fixation to form HMG CoA. This was split by an HMG CoA cleavage enzyme to acetyl CoA and free acetoacetic acid (12). The reverse of the cleavage reaction did not appear to take place readily.

It has hitherto been uncertain as to whether acetyl CoA condensed with free acetoacetate or with AcAc CoA in the primary condensation reaction leading to branched chain fatty acid synthesis (Reaction 2). In this paper evidence is presented that acetyl CoA and AcAc CoA are the reacting moieties in the formation of HMG. Studies on the cellular distribution of the enzyme show that the enzyme system is located chiefly in the microsomes. The enzyme catalyzing the formation of HMG from acetyl CoA and AcAc CoA has been designated the HMG-condensing enzyme to distinguish it from the well known condensing enzyme catalyzing the formation of citric acid (13).²

Methods and Materials

Preparation and Fractionation of Rat Liver Homogenate—All operations were carried out at 4° unless otherwise indicated. Freshly removed rat livers were finely minced for 30 seconds with four razor blades set 6 mm apart in an aluminum block. 2 ml of medium as prepared by Rabinowitz and Gurin (6) were added per gm of liver and the suspension was homogenized for 45 seconds in a loose fitting homogenizer (a Teflon pestle having

² The crystalline citrate-condensing enzyme is inactive with acetyl CoA and acetoacetyl CoA as substrates as measured optically by the disappearance of acetyl CoA thio ester absorption at $240 \text{ m}\mu$ and the enolate absorption of acetoacetyl CoA at $310 \text{ m}\mu$ (personal communication from Dr J. R. Stern).

an outside diameter of 25 mm and a glass mortar with an inside diameter of 27 mm). When 200 ml of homogenate were collected, the cell debris was removed by centrifugation at $100 \times g$ for 10 minutes. Mitochondria were removed by centrifugation at $5000 \times g$ for 30 minutes. Microsomes were obtained by centrifugation at $100,000 \times g$ in a Spinco preparative ultracentrifuge for 30 minutes. The supernatant solution is referred to in the text as the supernatant fraction. The microsomal pellet was homogenized for 30 seconds with 2 volumes of 10^{-3} M Versene and allowed to stand at 0° for 5 minutes, then centrifuged at $100,000 \times g$ for 30 minutes, and the supernatant solution is referred to in the text as the microsomal extract. The microsomal extract could be lyophilized and the dried powder would remain stable for 1 to 2 weeks when stored at -15° after which it gradually lost enzymatic activity.

Preparation of HMG-Condensing Enzyme from Beef Liver—All operations were at 4° . A beef liver obtained directly from the slaughterhouse was packed in ice and processed as quickly as possible. The liver was ground with a meat grinder to a fine paste. 100 gm of liver paste and 200 ml of Rabinowitz-Gurin medium were homogenized in a Waring blender for 30 seconds at one-third the normal line voltage. The homogenate was centrifuged in 1 liter buckets in an International PR-2 centrifuge for 10 minutes at 2000 r.p.m. to remove cell debris. The supernatant solution was centrifuged at $5000 \times g$ for 30 minutes to remove mitochondria. The resultant supernatant fluid was centrifuged in a Spinco preparative ultracentrifuge for 30 minutes at $50,000 \times g$ to obtain microsomes. The microsomal pellet was suspended in Rabinowitz-Gurin medium equal to one-half the original homogenate volume and recentrifuged at $50,000 \times g$. The washed microsomal pellet was homogenized in a Potter-Elvehjem homogenizer for 1 minute with 5 volumes of 10^{-3} M Versene. After standing for 5 minutes it was centrifuged at $50,000 \times g$ for $\frac{1}{2}$ hour. The supernatant solution was lyophilized and could be stored at -15° for 1 to 2 weeks without any loss of activity.

The lyophilized preparation was purified about 10-fold further as follows. 400 mg of powder in 10 ml of H_2O were brought to pH 5.0 with acetic acid and the precipitate was discarded. Ammonium sulfate was added to 45 per cent saturation (calculated on saturation at 25°) and the precipitate was dialyzed against 10^{-3} M Versene for 3 hours. During the dialysis some inactive protein precipitated from solution and this was removed. The dialyzed solution could be kept frozen for 1 week without loss of activity. It steadily lost activity thereafter. This preparation could be purified further by adsorption of inactive protein on calcium phosphate gel (2 mg of gel per 25 mg of protein).

Preparation of Cell-Free Yeast Extract—27 gm of Red Star bakers' yeast were suspended in 48 ml of 2.5×10^{-3} M potassium phosphate buffer,

pH 6.75, at 0°. The cells were disintegrated with a Nossal shaking apparatus (14). Two shaking periods of 15 seconds were used in conjunction with 5 ml of No. 12 Ballotini beads (obtained from the Minnesota Mining and Manufacturing Company). Cell debris and beads were removed by centrifugation at $20,000 \times g$ for 10 minutes. The supernatant solution was then centrifuged in a Spinco ultracentrifuge for 30 minutes at $105,000 \times g$. The resultant supernatant solution was used as the condensing enzyme. It retained its activity for 1 week when stored at -15° . The $105,000 \times g$ pellet also exhibited condensing enzyme activity. It is possible that in the intact yeast cell all of the HMG synthesis occurs in these particles and that the activity seen in the supernatant solution results from damage to the particles. Klein and Booher (15) have found that most of the sterol synthesis in yeast is localized in these particles.

Preparation of Substrates and Materials—CoA was a commercial preparation from the Pabst Laboratories. Acetyl CoA was prepared from acetic anhydride by the general method of Simon and Shemin (16), acetic- C^{14} anhydride was obtained from commercial sources. AcAc CoA was prepared from diketene by the method of Wieland and Rueff (17). Acetyl and acetoacetyl derivatives of pantetheine were prepared in a similar manner. The pantetheine obtained commercially (Nutritional Biochemicals Corporation) was reduced by sodium borohydride.

Acetoacetate-3- C^{14} was prepared by the method of Curran (18). HMG was prepared by the method of Adams and Van Duren (19) and β -hydroxyisovaleric acid as the Ag salt by the procedure of Kohn (20). Potassium acetoacetate was prepared by incubating redistilled ethyl acetoacetate with an equivalent amount of 1 N KOH for 24 hours at 4° and titrating to neutrality, followed by lyophilization. Water was then added to bring to a 1.0 M concentration and the solution was stored at -15° .

Enzymatic Assay—The labeled substrates were incubated with the enzyme preparations and additions as described in the footnotes to Tables I to VI, then 6 N KOH was added to a final concentration of 0.2 N and the mixture was allowed to stand for 30 minutes at room temperature to hydrolyze acyl CoA derivatives. Since these intermediates occur in amounts too small to be measured and isolated by ordinary procedures, 100 μ moles of HMG and 200 μ moles of β -hydroxyisovaleric acid or β -hydroxybutyric acid were added as carrier, the mixture was acidified to Congo red with H_2SO_4 , taken up in Celite (2 gm of Celite per ml of mixture), and continuously extracted with ether for 4 to 6 hours. After removal of the ether the extract was acidified to Congo red with 1 N H_2SO_4 . 2 mmoles of acetic acid were added and the solution was steam-distilled to 10 times the original volume. The distillate contained labeled acetic acid and other steam-volatile acids. Acetoacetic acid is destroyed by this procedure.

The residue which contained HMG, β -hydroxyisovaleric acid, β -hydroxybutyric acid, and β -methylglutaconic acid was placed on a Dowex 1 formate column (1.1 \times 130 cm) and eluted with 0.1 N formic acid. The fraction from 30 to 90 ml contained β -hydroxybutyrate, and β -hydroxyisovalerate, and was saved for further purification. HMG was invariably obtained in the fraction from 265 to 415 ml in yields ranging from 50 to 75 per cent. The fraction containing HMG was evaporated to dryness under an infrared lamp with care taken not to exceed 80°. After evaporation a colorless syrup of HMG was obtained which crystallized readily in small rosettes. The HMG was titrated with KOH and aliquots containing 8 μ moles of the dipotassium salt were placed on stainless steel planchets and evaporated, the radioactivity was counted in a gas flow counter. The specific activity in counts per minute per micromole was multiplied by the number of micromoles of carrier added to obtain total activity.

The fraction containing β -hydroxybutyrate and β -hydroxyisovalerate was steam-distilled to remove formate and titrated to neutrality with KOH. The K salts were evaporated to dryness, taken up in 0.6 ml of 3 N H_2SO_4 , mixed thoroughly with 1 to 2 gm of Celite No. 535, and packed on a column 2.0 cm in diameter consisting of 20 gm of Celite No. 535 thoroughly mixed with 10.0 ml of 0.2 N H_2SO_4 . Elution was carried out in the following sequence with solvents equilibrated with 0.2 N H_2SO_4 . First 200 ml of 100 per cent chloroform were passed through, followed by 95 per cent chloroform and 5 per cent butanol. β -Hydroxyisovalerate was obtained in the fraction from 30 to 80 ml with the second solvent and β -hydroxybutyrate in the fraction from 200 to 300 ml. The amount of these acids was readily determined by titration with brom thymol blue as indicator. 8 μ moles of the potassium salts were evaporated in planchets and the radioactivity was measured in a gas flow counter. Total activity was calculated in the same manner as that for HMG. Proteins were determined by the biuret procedure (21) and the Folin phenol reagent (22).

RESULTS AND DISCUSSION

Incorporation of Labeled Acetate into HMG, β -Hydroxyisovalerate, and β -Hydroxybutyrate in Various Intracellular Fractions—The incorporation of labeled acetate in HMG, β -hydroxyisovalerate, and β -hydroxybutyrate was determined in various cellular fractions prepared as described. The results of representative experiments are shown in Table I. It will be noted that the whole homogenate of rat liver after removal of cell nuclei and debris converts a large part of the labeled acetate to β -hydroxybutyrate. Similar results were obtained by Katz and Chaikoff (23) with liver slices. The supernatant fraction devoid of particulate matter is also able to incorporate acetate into the three acids tested, but more acetate accumulates

as HMG and β -hydroxyisovalerate and less as β -hydroxybutyrate. Apparently the presence of mitochondria favors β -hydroxybutyrate synthesis, possibly by providing a source of reduced DPN for reduction of acetoacetate to β -hydroxybutyrate. Microsomes or mitochondria suspended in phosphate buffer are incapable of synthesizing HMG and β -hydroxyisovalerate under these conditions although mitochondria can form β -hydroxybutyrate. When the supernatant fraction is added to microsomes (Experiment 1), a marked decrease in the isotope incorporated into β -hydroxyiso

TABLE I
Incorporation of $C^{14}H_3COOH$ into HMG, β -Hydroxyisovalerate, and β -Hydroxybutyrate by Various Cellular Fractions of Rat Liver

Experiment No		HMG	β Hydroxyisovalerate	β Hydroxybutyrate
1	Whole homogenate	775	1,600	13,800
	Supernatant fraction (100,000 \times g)	2350	11,500	3,500
	Microsomes	12	225	75
	Mitochondria	112	75	2,020
	Supernatant + mitochondria	120	125	16,100
	“ + microsomes	1480	1,075	4,570
2	Whole homogenate	700	350	54,000
	Homogenate minus mitochondria	775	3,820	8,700
	Supernatant fraction (100,000 \times g)	1400	5,750	3,500
	Microsomes	0	0	0
	Microsomal extract + supernatant fraction	2150	4,500	6,850

The homogenate of rat liver was prepared as described under "Methods and materials." The individual cellular fractions from 30 ml of homogenate were suspended in Rabinowitz-Gurin medium to a volume of 60 ml. The final volume was 62 ml. Each test tube contained 1 μ mole of ATP, 0.1 μ mole of CoA, 4 μ moles of $C^{14}H_3-COONa$ (1.75×10^5 c.p.m. per μ mole). The incubation was for 2.5 hours at 38° in air. The figures represent total counts per minute in the respective acids.

valerate and β -hydroxybutyrate is noted. This may indicate a utilization of β -hydroxyisovalerate for cholesterol synthesis since Bucher and McGarahan (24) have shown that cholesterol synthesis takes place chiefly in the microsomes and Rabinowitz and Gurin (5) found that β -hydroxyisovalerate may be used for this purpose.

A combination of the extract from microsomes lysed in 10^{-3} M Versene and the supernatant fraction from the homogenate caused more acetate to accumulate in HMG to a greater extent than that observed in the supernatant fraction alone (Experiment 2). This effect was investigated in greater detail and the results are shown in Table II. These experiments show that the microsomal extract contains the enzymes which synthesize

HMG from acetate, ATP, and CoA. When the supernatant fraction is added, the acetate is shifted from HMG towards β -hydroxyisovalerate and β -hydroxybutyrate synthesis. Apparently the microsomal extracts do not have the ability to form β -hydroxyisovalerate and therefore have lost the enzyme which decarboxylates HMG or some derivative of HMG to β -hydroxyisovalerate. This extract has an absolute dependence on ATP and CoA for HMG synthesis. It will be seen later that this dependence results from a requirement of the acetate-activating enzyme for these factors since acetyl CoA does serve as the substrate in the absence of ATP and CoA.

The presence of the acetate-activating enzyme in this system appears to

TABLE II
*Incorporation of ^3C H_3COOH into HMG, β -Hydroxyisovalerate,
and β -Hydroxybutyrate by Microsomal Extracts*

Experi- ment No.		HMG	β -Hy- droxy- isoval- erate	β -Hy- droxy- butyrate
1	Supernatant fraction	570	2300	3,650
	Microsomal extract	2,900	300	50
	" " — supernatant fraction	1,170	1650	14,400
2	Supernatant fraction	725	5750	2,850
	Microsomal extract	6,100	300	0
	" " — supernatant fraction	1,150	5600	5,750
3	" " "	17,000		
	" " extract — CoA	1,600		
	" " extract — ATP	250		

Conditions and additions the same as those for Table I.

be due to the small amount of supernatant fraction adhering to the microsomes. It was found that prewashing the microsomes led to inactivity with respect to synthesis of HMG from acetate, ATP, and CoA, although activity with acetyl CoA as substrate was unimpaired. When unwashed microsomes were lysed with Versene, a preparation capable of incorporating acetate almost exclusively into HMG was obtained. Since an addition of large amounts of particle-free supernatant fraction causes a greater proportion of isotope to accumulate in β -hydroxyisovalerate and β -hydroxybutyrate, it appears that the amount of supernatant fraction adhering to the microsomal pellet fortuitously provided enough enzyme for the activation of acetate but was still limiting with respect to the other enzymes converting acetyl CoA to β -hydroxyisovalerate and β -hydroxybutyrate. These findings are in agreement with those of Bucher and McGarrahan.

(24) who also found that a supernatant fraction is necessary for acetate activation

When the microsomal fraction was compared with various cellular fractions, it was found to have the highest activity in the synthesis of HMG from acetate (Table III). Since the total activity in the microsomal preparation is so much larger than that of the original homogenate and the other cellular fractions, it is obvious that the synthesis of HMG in the cruder fractions is either inhibited because of competition for acetyl CoA by other pathways, or that HMG is metabolized to other compounds. In any case it appears that the microsomes contain the highest concentration of the enzyme system which synthesizes HMG. This observation

TABLE III
Activity of Various Cellular Fractions of Rat Liver with
Respect to Incorporation of Acetate into HMG*

	Specific activity	Total activity†
Whole homogenate	10	54,000
Soluble preparation of Rabinowitz-Gurin (6)	20	100,000
Supernatant fraction	30	150,000
Unwashed lysed microsomal preparation + cysteine	8000	800,000

* A unit of activity is expressed as the counts per minute incorporated into HMG per mg of protein when 4 μ moles of $C^{14}H_3COOH$, 3 μ moles of ATP, and 0.3 μ mole of CoA are incubated under the conditions described in Table I. Only the microsomal preparation was limiting with respect to cysteine and 10 μ moles of cysteine were therefore added. The specific activity of the acetate was 1.75×10^5 c.p.m. per μ mole.

† Total activity represents the total number of units present in the various fractions as derived from 100 gm of rat liver.

is in agreement with the postulate that HMG or a metabolite thereof might be an intermediate in cholesterol synthesis, in view of the establishment of the microsomes as the chief site of cholesterol synthesis (24). The unwashed microsomal extract from rat liver also utilized acetyl- C^{14} CoA and acetyl- C^{14} pantetheine for the synthesis of HMG. In addition it could form HMG from acetoacetate, ATP, and CoA, indicating that an acetoacetate-activating enzyme is also present (25, 26).

Nature of 4-Carbon Unit Which Condenses with Acetyl CoA—The following experiments with labeled acyl CoA derivatives show that AcAc CoA is the 4-carbon compound which reacts with acetyl CoA to form HMG.

The first evidence was obtained when unlabeled acetyl CoA was incubated with labeled acetoacetate and labeled acetyl CoA was incubated with unlabeled acetoacetate. The results are shown in Table IV. When an

unwashed rat liver microsomal extract was incubated with C^{14} -acetoacetate and unlabeled acetyl CoA, there was no significant increase in the amount of C^{14} incorporated into HMG above the control values (Tube 1 compared to Tube 2). On the other hand, when ATP was present, the incorpora-

TABLE IV
*Incorporation of Acetoacetate and Acetoacetate- C^{14} into HMG
by Rat Liver Microsomal Extract*

Tube No		Total c p m HMG						
		Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6	Experiment 7
1	3- C^{14} -Acetoacetate, no addition	125	75	78	145			
2	3- C^{14} -Acetoacetate + acetyl CoA			150	85			
3	3- C^{14} -Acetoacetate + acetyl CoA + ATP			1300	1500			
4	3- C^{14} -Acetoacetate + ATP + CoA SH	3000	1140	4150				
5	Acetyl- C^{14} CoA, no addition					4000	2120	4000
6	“ “ + 10 μ moles acetoacetate					4400	2250	4200
7	Acetyl- C^{14} CoA + 30 μ moles acetoacetate					4100	2750	4250
8	Acetyl- C^{14} CoA + 100 μ moles acetoacetate					4000	2240	4600

In Experiments 1 and 2 each tube contained 15 μ moles of acetoacetate- C^{14} (4×10^4 c p m per μ mole), 10 mg of bovine albumin, 15 mg of lyophilized rat liver microsomal extract, 220 μ moles of phosphate buffer, pH 7.0, 10 μ moles of Mg^{++} , and 60 μ moles of nicotinamide. Final volume 2.5 ml. Incubation 2.5 hours at 37° in air. In Experiments 3 to 7 each tube contained 8 mg of lyophilized rat liver microsomal extract, 10 mg of bovine albumin, 10 μ moles of cysteine, 10 μ moles of Mg^{++} , 220 μ moles of phosphate buffer, pH 7.0. Tubes 1 to 4 in Experiments 3 and 4 contained 10 μ moles of acetoacetate-3- C^{14} (4×10^4 c p m per μ mole). Tubes 5 to 8 in Experiments 5, 6, and 7 contained 0.3 μ mole of acetyl- C^{14} CoA (carboxyl-labeled) (5.5×10^5 c p m per μ mole). The additions consisted of 0.3 μ mole of acetyl CoA, 0.2 μ mole of CoA SH, 2.0 μ moles of ATP, and free acetoacetate as indicated. Final volume 2.5 ml, incubated in air for 2 hours at 37°.

tion of C^{14} was greatly stimulated (Tube 3). An even greater incorporation was observed with ATP, CoA, and C^{14} -acetoacetate (Tube 4). When acetyl- C^{14} CoA was used (Tube 5), there was good incorporation of C^{14} , proving that HMG is synthesized by the system. These observations show that free acetoacetate is not a substrate for the formation of HMG, since in the presence of acetyl CoA no isotope from C^{14} -acetoacetate was incor-

requirements for acetyl CoA. Since β -ketothiolase is present, the addition of AcAc CoA together with endogenous CoA SH would form more acetyl CoA. Although the final specific activity of the acetyl CoA pool would undoubtedly be diluted, the effect of saturating the enzyme system might compensate for the dilution and thus more counts would be incorporated into HMG. The results obtained in Experiment II, Table V, indicate that even when the saturation level of acetyl CoA has been reached the addition of AcAc CoA again leads to a greater incorporation of isotope. The activating effect of AcAc CoA is fully explained by assuming that it is the reacting substrate and that higher concentrations saturate the condensing enzyme enabling it to react more efficiently with acetyl CoA to form HMG. In effect the β -ketothiolase reaction which is essential when acetyl CoA is the only substrate is now bypassed when sufficient AcAc CoA is present.

The amount of HMG formed is actually greater than that indicated by the amount of C^{14} incorporated because of the following factor. When acetyl- C^{14} CoA is the only substrate, 3 carbons of HMG will be labeled (1). On the other hand, when acetyl- C^{14} CoA and AcAc CoA are the substrates, a large part of the HMG will have only 1 carbon labeled, *i.e.* that supplied by the labeled acetyl groups of acetyl- C^{14} CoA, and in addition the acetyl- C^{14} CoA pool will be diluted if β -ketothiolase and free CoA SH are present.

Johnston *et al* (7) working with a flaxseed extract indicated that free acetoacetate rather than AcAc CoA was the 4-carbon substrate for HMG synthesis on the following basis. They found that the addition of acetoacetate together with ATP and CoA decreased acetate- C^{14} incorporation into HMG by 80 per cent, yet the incorporation of acetate into citrate was decreased only 20 per cent. They reasoned that, if AcAc CoA were formed, it would be converted to acetyl CoA and would decrease the incorporation of isotope into citrate to the same extent as that observed for HMG. Their observation, however, can also be interpreted to mean that AcAc CoA is the substrate, since in the case of citrate the observed dilution is directly related to the dilution of the acetyl CoA pool only. However, in the case of HMG the observed decrease in isotope incorporation is due not only to the dilution of the acetyl CoA pool, but also to the dilution of the AcAc CoA pool which would be formed from acetoacetate, ATP, and CoA, hence, it would be expected that the decrease in radioactivity observed in citrate would be much less than that found for HMG.

Conditions might also be conceived in which if the β -ketothiolase were present in great excess relative to the HMG-condensing enzyme the addition of acetoacetyl CoA to acetyl- C^{14} CoA would tend to reduce the incorporation of isotope into HMG because of the rapid conversion of acetoacetyl

CoA to acetyl CoA and consequent dilution of the acetyl- C^{14} CoA pool. This is actually observed in preparations of lyophilized microsomal extracts prepared from beef liver prior to ammonium sulfate fractionation (Table VI). After purification, however, the reverse is observed and the increased activity with acetoacetyl CoA is obtained.

The fact that labeled free acetoacetate is not incorporated into HMG, plus the subsequent observations concerning the effect of AcAc CoA and free acetoacetate, offers strong support for the hypothesis that acetyl CoA and acetoacetyl CoA are the reacting moieties in the condensation reaction responsible for HMG synthesis. The elucidation of the nature of the products of this condensation, i.e. whether a mono- or di-CoA derivative

TABLE VI

Effect of AcAc CoA with Crude and Purified Extracts of Beef Liver Microsomes

	Total c p m in HMG
Crude extract + 1.0 μ mole Ac- C^{14} CoA	480
" " + 1.0 " " " + 0.5 μ mole AcAc CoA	300
Fraction I + 1.0 μ mole Ac- C^{14} CoA	163
" I + 1.0 " " " + 0.5 μ mole AcAc CoA	200
" II + 1.0 " " " "	81
" II + 1.0 " " " + 0.5 μ mole AcAc CoA	400

The crude extract contained 0.35 mg of protein and refers to the lyophilized microsomal extract. Fraction I contained 0.24 mg of protein and refers to the 0 to 45 per cent ammonium sulfate fraction. Fraction II contained 0.18 mg of protein and refers to the calcium phosphate-treated ammonium sulfate fraction. Each tube contained 10 mg of bovine albumin, 220 μ moles of PO_4 buffer, pH 7.0, and 10 μ moles of Mg^{++} . The incubation was for 2 hours at 38° in air. The specific activity of the acetyl- C^{14} CoA was 5.5×10^6 c p m per μ mole.

of HMG is formed, must await purification of the condensing enzyme free of β -ketothiolase and other interfering enzymes. Also the relationship of the product to subsequent intermediates in the pathway of cholesterol biosynthesis requires further investigation.

The evidence in the preceding section that isotope from labeled acetic acid can be shifted from fatty acid cycle intermediates (such as β -hydroxybutyrate) on one hand to presumed steroid precursors HMG and β -hydroxyisovalerate points to the central role played by AcAc CoA in that it is the focal point at which fatty acid and steroid metabolism may be linked. Four enzymes may play an important role in determining the shift of acetyl units from fatty acids to sterols: firstly, the β -ketothiolase and the supply of free CoA SH, secondly, the β -ketoreductase and the supply of reduced diphosphopyridine nucleotide (28), thirdly, the HMG-condensing enzyme

which serves to initiate the synthesis of branched chain fatty acid intermediates by utilizing acetyl CoA and AcAc CoA, two key intermediates in fatty acid metabolism, and, finally, the AcAc CoA deacylase (29). The interplay of all of these enzymes on AcAc CoA would undoubtedly influence steroid metabolism and this point requires further investigation. The relationship of the reaction of active CO_2 and β -hydroxyisovaleryl CoA to form HMG CoA and the role of the HMG CoA cleavage enzyme of Bachhawat *et al* (11, 12) to the HMG-condensing enzyme system is presently obscure and awaits further work for clarification.

SUMMARY

Rat liver and beef liver homogenates and cell-free yeast preparations were fractionated by centrifugal techniques and the incorporation of acetate- C^{14} into β -hydroxy- β -methylglutaric acid, β -hydroxyisovaleric acid, and β -hydroxybutyric acid by various cellular fractions was determined. It was found that liver microsomes contain the major part of the enzyme system which forms β -hydroxy- β -methylglutaric acid. The particle-free supernatant fraction contained enzyme systems and reduced substrates which shifted acetate from β -hydroxy- β -methylglutaric and β -hydroxyisovaleric acids to β -hydroxybutyric acid formation. Extracts of microsomes were prepared which could synthesize β -hydroxy- β -methylglutaric but not β -hydroxyisovaleric acid and β -hydroxybutyric acid. With these extracts and the use of labeled substrates such as acetyl- C^{14} coenzyme A (CoA), $\text{CH}_3\text{-C}^{14}\text{O-CH}_2\text{COOH}$, and acetoacetyl CoA it was determined that the 4-carbon moiety which condenses with acetyl CoA to form β -hydroxy- β -methylglutaric acid is acetoacetyl CoA and not free acetoacetate. The implications of this finding are discussed with reference to sterol and fatty acid metabolism.

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THE METABOLIC REDUCTION OF ORGANIC NITRO GROUPS*

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The reduction of organic nitro groups is a well known metabolic reaction, but the nature of the enzymes effecting this reaction is unknown. Westfall reported (1) that liver, kidney, and heart are most active in the reduction of trinitrotoluene, and that a succinic dehydrogenase preparation from beef heart is capable of effecting this reaction (2). However, Bueding and Jolliffe (3) found that succinic dehydrogenase could not catalyze this reaction directly, they postulated that such substrates as succinate, lactate, and malate regenerated DPNH,¹ which in turn reduced trinitrotoluene catalytically through a flavoprotein. Purified xanthine oxidase can reduce organic nitro groups (3-5) as well as inorganic nitrate (6, 7), liver xanthine oxidase (5) reduced nitrophenols with DPNH as electron donor.

Since xanthine oxidase is a molybdenum-containing enzyme and since other enzymes capable of reducing nitrate contain Mo (7), the purpose of the present study was to assess the role of Mo enzymes in the reduction of organic nitro groups. This was done by the administration of tungstate as a means of removing Mo and Mo enzymes from the tissues (8). The specificity of the tungstate effect was controlled by overcoming it with dietary molybdate. In this way it has been shown that most, if not all, of the enzymes in rat tissues capable of reducing the nitro group of *p*-nitrobenzene sulfonamide are Mo-dependent enzymes. From additional fractionation and substrate studies it is also evident that animal tissues contain a number of different enzymes capable of reducing organic nitro groups and that any one system exhibits some specificity toward different nitro substrates.

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¹ The following abbreviations are used: DPN, diphosphopyridine nucleotide, DPNH, reduced DPN, TPNH, reduced triphosphopyridine nucleotide, *p*NBSA, *p*-nitrobenzene sulfonamide, FAD, flavin adenine dinucleotide, FMN, flavin mononucleotide, Mo, molybdenum, W, tungsten.

Microorganisms are also capable of reducing organic nitro compounds (9-12). However, the system in *Aspergillus niger* which reduces *p*NBSA was found to be independent of Mo.

Methods

In Vivo—Normal adult rats previously maintained on chow were fed a purified 24 per cent casein diet (13), modified as follows, for 2 to 4 weeks: (1) 24 per cent Labco casein (as previously described), (2) 24 per cent casein plus 72 mg of Na_2WO_4 per kilo, (3) 24 per cent casein plus 72 mg of Na_2WO_4 per kilo + 50 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ per kilo, (4) 0 per cent protein, the casein being replaced with glucose. At an average of 22 or 25 days, each rat was placed in an individual metabolism cage and three 24 hour urine samples were collected under toluene. The first was a control sample which provided an estimation of naturally occurring diazotizable substances in rat urine (these were appreciably lower on the protein-free diet), the second was collected after a suspension of 10 mg of *p*NBSA in 5 ml of H_2O was given each rat by stomach tube, and the third 24 hour urine sample was collected during the period of 24 to 48 hours after the *p*NBSA administration. Each specimen of urine was analyzed for free and total sulfanilamide by the Bratton and Marshall procedure (14), the unchanged nitro compound was determined by the zinc reduction procedure of Flynn and Kohl (15). The corresponding values for the control urine were subtracted from each of the experimental samples, and from these corrected figures the data were calculated as (a) per cent of total excretory products in the reduced form as sulfanilamide and as (b) per cent of the sulfanilamide excreted free and acetylated.

In Vitro—At the conclusion of the *in vivo* studies, each rat was killed and the liver was analyzed for xanthine oxidase (16) and for its ability to reduce *p*-nitrobenzene sulfonamide *in vitro*. The same liver homogenate, prepared with 5 volumes of 0.04 M phosphate buffer, pH 7.4, was used in both determinations. 4 ml of the homogenate were placed in the body of a Thunberg tube, 1 ml of a solution containing 1 mg of *p*NBSA in 0.1 M phosphate buffer, pH 7.5, was placed in the side arm. After being evacuated, filled with N_2 , and equilibrated at 37.5°, the solutions were mixed and incubated for either 15 or 30 minutes. In some cases, purified milk xanthine oxidase or Na_2MoO_4 was added to additional aliquots of the liver homogenate, and the tubes were incubated for 30 minutes. All reactions were stopped by the addition of 5 ml of 10 per cent trichloroacetic acid, and the centrifuged supernatant fluids were analyzed for free sulfanilamide.

In additional *in vitro* studies (Series II), the Thunberg tube contained 0.67 gm of fresh liver (or its equivalent) plus 1 mg of *p*NBSA and additional factors in a total volume of 5 ml of buffered solution, the tube was incubated anaerobically for 30 minutes at 37.5°, and free sulfanilamide was

determined on a trichloroacetic acid filtrate. As indicated under "Results," 3.3 mg of DPNH or TPNH, 0.15 ml of 1 M succinate, 0.05 M hypoxanthine, 3 M acetaldehyde, or 1 ml of 0.01 M glutathione was added to the tubes. In some experiments, the liver homogenate was centrifuged in a Spinco preparative ultracentrifuge (rotor No. 40) at 40,000 r.p.m. for 30 minutes. The supernatant fraction was tested directly. The precipitate was suspended in buffer, centrifuged again for 30 minutes, and resuspended in fresh buffer before being tested. In comparing the effects of various diets, the precipitate was fortified with 6.4 mg of DPNH as the

TABLE I

Effect of Tungstate Feeding and Protein Depletion for 22 Days on Reduction in Vivo and in Vitro of p-Nitrobenzene Sulfonamide

Diet	No. of rats	Body weight, gm	24 hr urinary excretions after 10 mg pNBSA			Reduction <i>in vitro</i> of pNBSA, γ amine formed per 667 mg liver		Liver xanthine oxidase determination, c mm O ₂ per 20 min per 282 mg
			Total NO ₂ + NH ₂ compounds, mg	Per cent reduced	Per cent sulfanilamide free	15 min	30 min	
Chow	11	361	5.39* ±0.24	83.3 ±2.3	14.5 ±0.6	173 ±6.5	300 ±6.2	31 ±2.0
24% casein	5	223	5.21 ±0.46	77.9 ±2.0	16.7 ±0.7	136 ±5.5	232 ±10.0	25 ±1.1
Tungstate	5	185	4.27 ±0.70	65.9 ±3.0	23.2 ±1.0	32 ±4.9	60 ±9.5	0
" + Mo	8	298	4.97 ±0.24	76.5 ±2.1	17.5 ±1.0	159 ±7.0	267 ±10.2	20 ±1.6
0% protein	3	99	4.47 ±0.47	62.2 ±5.2	20.0 ±1.6	49 ±5.6	86 ±8.7	0
0% "	3	99				110 ±8.5	197 ±20.2	21 ±5.8

* Mean ± standard error

electron donor substrate. Concentrated liver homogenates were dialyzed for 24 hours in the cold against several changes of phosphate buffer containing 1×10^{-4} M cysteine. The dialyzed preparation was tested with and without the above substrates. Whole heart homogenates were tested directly without supplementation.

Results

In Vivo—The urinary excretions during the first 24 hours after pNBSA was given are shown in Table I. The total urinary metabolites (nitro plus amino compounds) amounted to about 5 mg, of which approximately 80

per cent was in the reduced form for the normal rats fed chow or a purified 24 per cent casein diet. Rats on the tungstate and zero protein diets excreted only 62 to 66 per cent of the urinary metabolites in the reduced form. Dietary molybdate largely reversed the metabolic defect produced by tungstate, and allowed over 75 per cent reduction of the excretory products.

The 24 to 48 hour urinary metabolites were not particularly informative since over 90 per cent of the total was in the reduced form and 86 to 90 per cent was acetylated in all groups. The chow-fed rats excreted approximately 0.3 mg. of the total products, or 6 per cent of the first 24 hour excretion. The 24 per cent casein and tungstate plus molybdate groups excreted a corresponding 13 per cent, whereas the tungstate and protein-deficient groups excreted 17 per cent.

The tungstate-fed and protein-depleted rats acetylated only 77 to 80 per cent of the sulfanilamide formed by this reduction, while the rats on the other diets acetylated 82 to 85 per cent. This suggested a possible defect in the acetylation reaction as well as in the reduction of the nitro group as a result of feeding tungstate. Additional studies were therefore conducted in which both sulfanilamide and *p*NBSA were fed to the same rats at different times. The results shown in Table II were comparable with Series I inasmuch as the tungstate-fed rats excreted only 62 per cent of the urinary metabolites in the reduced form as compared with 80 per cent for the chow and 70 per cent for tungstate plus molybdate diets. The amount of the reduction product which was acetylated was again 83 per cent for the control rats and only 76 per cent for the tungstate-fed rats. However, when sulfanilamide itself was administered, all three dietary groups excreted about 80 per cent of the administered dose and acetylated approximately 60 per cent of the urinary products. Hence tungstate feeding had no effect on the acetylation reaction itself, and the effect observed in both *p*NBSA experiments must be related indirectly to the effect of tungstate on the reduction reaction.

In Vitro—The results of the *in vitro* studies are also shown in Tables I to III. While the chow-fed rats had a liver xanthine oxidase of 31 and formed 300 γ of sulfanilamide from the nitro analogue in 30 minutes, the tungstate-fed rats had no liver xanthine oxidase by the usual manometric determinations² and formed only 60 γ of the amine. Addition of molybdate to the tungstate diet restored the liver xanthine oxidase to 20 and the amine formation to 267. The protein-depleted rats were grouped into those with no liver xanthine oxidase forming 86 γ of amine and those retaining an average of 21 for liver xanthine oxidase and forming 197 γ .

² Small amounts of xanthine oxidase can be demonstrated in such livers by measuring allantoin production.

of amine The addition of 100 γ of Mo (as Na_2MoO_4) to the Thunberg tube had no effect (± 5 per cent) on the reduction *in vitro* of the nitro group by any of these livers, and did not restore the xanthine oxidase activity of the livers in the tungstate or protein-depleted groups

Purified milk xanthine oxidase plus 0.15 ml of 0.05 M xanthine or hypoxanthine, instead of liver homogenate, in the anaerobic Thunberg procedure produced very little sulfanilamide An amount of milk xanthine

TABLE II

Effect of Feeding Tungstate or Tungstate Plus Molybdate Diet for 24 Days on Reduction in Vivo and Acetylation of 10 Mg of Oral Dose of p-Nitrobenzene Sulfonamide or Sulfanilamide and on Reduction in Vitro of pNBSA by Liver and Heart Homogenates

Diet	Body weight	Liver xanthine oxidase	Urine excretion after 10 mg orally					Reduction <i>in vitro</i> of pNBSA†			
			p Nitrobenzene sulfonamide			Sulfanilamide		Liver, 0.67 gm			0.5 gm heart homogenate
			Total $\text{NH}_2 + \text{NO}_2$	Reduced	Sulfanilamide free	Total sulfanilamide	Free	Homogenate	Supernatant solution	Ppt + DPNH	
	gm		mg	per cent	per cent	mg	per cent				
Chow	218	29	4.89*	79.5*	16.8*	7.48*	40.2*	333	120	76	63
			± 0.14	± 1.3	± 1.4	± 0.30	± 2.3	± 9	± 7	± 4	± 4
Tungstate	210	0	4.95	61.9	24.4	8.20*	41.1*	55	34	22	23
			± 0.19	± 2.1	± 1.0	± 0.04	± 1.4	± 4	± 3	± 3	± 3
" + molybdate	206	26	4.61	69.8	17.6	8.71*	41.5*	271	86	56	35
			± 0.35	± 0.6	± 0.8	± 0.13	± 1.3	± 7	± 5	± 5	± 3

* Mean \pm standard error for eleven or twelve rats, all the others are mean \pm standard error for five or six rats

† Recorded as micrograms of sulfanilamide formed from 1 mg of pNBSA in 30 minutes

oxidase, which utilized 65 to 85 c mm of O_2 per 20 minutes in the manometric assay, formed only 5 or 6 γ of sulfanilamide from an original 1000 γ of pNBSA in 30 minutes When added to the liver homogenate *in vitro*, an amount of milk xanthine oxidase which restored the liver xanthine oxidase of tungstate-fed or protein-depleted rats to the control level of 25 to 30 restored the nitro-reducing activity from 60 to approximately 100 (micrograms of sulfanilamide formed per 30 minutes per 0.67 gm of liver) When the nitro-reducing activity of the liver was high (230 to 300 γ), the addition of milk xanthine oxidase *in vitro* actually depressed this reduction about 15 per cent Whether pNBSA was reduced to an intermediate sub-

stance by the purified xanthine oxidase was not determined. However, little sulfanilamide was formed by this enzyme even in the presence of a crude homogenate.

A crude preparation of rat liver xanthine oxidase was made by high speed centrifugation of a homogenate to remove particulate matter and by precipitating the enzyme from the supernatant fluid between 30 and 60 per cent saturated ammonium sulfate. An amount of this preparation which gave an O_2 uptake of 50 c mm in 10 minutes with hypoxanthine substrate formed the following amounts of sulfanilamide, in micrograms, from *p*NBSA during 30 minute anaerobic incubation: no substrate 3, succinate 3, TPNH

TABLE III
Reduction in Vitro of pNBSA by Rat Liver Homogenates (Centrifuged or Dialyzed) Fortified with Various Substrates

	Normal rat liver, chow diet			Whole liver homogenate dialyzed 24 hrs		
	Whole-homogenate	Supernatant solution	Ppt	Chow diet	Tungstate diet	Tungstate + molybdate diet
Original homogenate	283	109	16	348	44	293
Dialyzed "				149	35	65
+ DPNH	317	141	103	329	75	219
+ TPNH	300	217	144	303	115	201
+ succinate	289	98	31	147	36	70
+ hypoxanthine	234 (1)	104	10 (3)	126	33	68
+ acetaldehyde	150 (2)	187	12 (4)	198	57	140

Recorded as micrograms of sulfanilamide formed from 1 mg of *p*NBSA in 30 minutes incubation with 0.67 gm of fresh liver homogenate or its equivalent of centrifuged supernatant fluid or precipitate. The corresponding values in the presence of glutathione were (1) 292, (2) 240, (3) 17, and (4) 25.

25, DPNH 55, hypoxanthine 50, acetaldehyde 117, DPNH plus hypoxanthine 91. Liver xanthine oxidase was therefore capable of reducing *p*NBSA with hypoxanthine substrate, but other enzymes active in this reaction might also be present in this preparation, the major reducing activity was not associated with the xanthine oxidase fraction.

Table III shows the effect of fortifying the liver homogenate with various potential substrates for the reduction of *p*NBSA. The original whole homogenate contained enough substrates so that only small effects were observed from additional electron donors. When separated into supernatant and precipitate fractions by centrifugation, the sum of the two individual activities was appreciably less than the original. This suggested a synergistic action between enzymes in the particulate and soluble fractions.

in the reduction of this organic nitro group by the whole homogenate. When the supernatant and precipitate fractions were recombined, three-fourths of the original activity was restored. The addition of boiled supernatant fluid to the precipitate fraction gave only slightly more activity than the precipitate alone. Both the supernatant and precipitate fractions utilized DPNH and TPNH as substrates for this reduction. Added succinate and hypoxanthine were relatively ineffective with all fractions, whereas acetaldehyde inhibited the whole homogenate and stimulated the supernatant fraction. Added glutathione generally increased the reduction by all fractions with all substrates slightly (approximately 10 per cent), but was most impressive in overcoming the inhibition of the whole homogenate and precipitate fraction by hypoxanthine and acetaldehyde.

Dialysis of the normal liver homogenate from chow-fed rats decreased its ability to reduce *p*NBSA by over one-half. The activity of the dialyzed preparation was restored almost to normal by DPNH or TPNH and was restored partially by acetaldehyde, succinate and hypoxanthine were without effect, citrate plus oxidized TPN, and to a lesser degree succinate plus TPN, were good electron donor substrates for this dialyzed liver homogenate. The dialyzed liver homogenate from tungstate-fed rats was much less active than normal, but responded to the various substrates in a similar manner. Tungstate feeding did not appear to remove the activity of the liver toward *p*NBSA in a selective manner in so far as these substrates were concerned.

The effect of tungstate feeding on the reduction of *p*NBSA by the supernatant and particulate fractions of liver is shown in Table II. The activity of both fractions was decreased comparably, but the synergistic effect of combining the two fractions, which was evident in both chow-fed and tungstate plus molybdate-fed rat livers, was much less impressive in tungstate-fed rats. Tungstate feeding also decreased the ability of heart homogenate to reduce *p*NBSA.

Other Species—Table IV shows the comparative reduction of *p*NBSA by several liver homogenates and their corresponding centrifugates when all were supplemented with DPNH. Not only did pigeon liver have an unusually high percentage of this activity localized in the sedimented particulate fraction, but a similar distribution and 85 per cent as much activity were obtained when either TPNH or acetaldehyde replaced DPNH as the electron donor substrate. By contrast, the soluble supernatant fraction of beef liver contained the bulk of this activity with either DPNH or acetaldehyde substrates, the latter gave about 75 per cent as much activity as the former, while hypoxanthine was only one-fourth as active.

The enzyme in the insoluble fraction of chicken liver was fractionated by digesting the high speed centrifuged precipitate with Pangelstin, precipitat-

ing the liberated enzyme with acetone, adsorbing an aqueous solution on $\text{Ca}_3(\text{PO}_4)_2$ gel, eluting it with 10 per cent saturated $(\text{NH}_4)_2\text{SO}_4$, and precipitating it with saturated $(\text{NH}_4)_2\text{SO}_4$. This enzyme preparation was relatively specific for DPNH substrate in the reduction of *p*NBSA, since little activity was obtained with TPNH, acetaldehyde, hypoxanthine, or succinate.

Comparative Reduction of 2,4-Dinitrophenol—A comparison of the relative rates of reduction of 2,4-dinitrophenol and *p*-nitrobenzene sulfonamide by livers from different species clearly indicated that different enzymes were involved. The reaction with 2,4-dinitrophenol was studied by incubating under nitrogen 4 ml of a phosphate buffer solution, pH 7.4, containing 1 ml of a 1:3 liver homogenate, with 320 γ of 2,4-dinitrophenol and 2.5 μ moles of DPNH or 5 μ moles of hypoxanthine for 30 minutes at 37.5°. After deproteinization with Na_2WO_4 and H_2SO_4 , 2 ml of the filtrate

TABLE IV

Reduction of pNBSA by Various Liver Fractions when Supplemented with DPNH

The recorded values are in micrograms of sulfanilamide formed per gm of original liver in 30 minutes

Liver	Original homogenate	Supernatant solution	Ppt
Rat	476	212	155
Beef	603	373	124
Chicken	568	286	237
Pigeon	344	65	246

were mixed with 1 ml of 0.5 M Na_3PO_4 and the optical density was measured at 400 m μ . The difference between the density of the incubated solution and a comparable non-incubated control was a measure of the amount of 2,4-dinitrophenol reduced, a change of 0.1 optical density unit was equivalent to 4.73 γ of 2,4-dinitrophenol per 3 ml.

Table V shows the comparative rates of reduction of 2,4-dinitrophenol and *p*NBSA by different tissue homogenates with added DPNH. Rat and chicken liver reduced both compounds well, but pigeon liver and beef kidney were much more effective with *p*NBSA than with 2,4-dinitrophenol. Similar, but less marked, differences were observed with rat and beef heart. Rat and chicken livers had the highest activity with 2,4-dinitrophenol and also contained the highest levels of xanthine oxidase or dehydrogenase. Since 2,4-dinitrophenol can be reduced by liver xanthine oxidase by use of DPNH substrate (5), some of the higher activity of these tissues can probably be attributed to the presence of the xanthine enzyme. However, since heart muscle does not contain xanthine oxidase and is able to reduce

2,4-dinitrophenol, one or more additional enzymes also catalyze this reaction

When chicken liver was fractionated (17) to purify the xanthine dehydrogenase and when 2.5 μ moles of DPNH were used as the substrate, the ability to form sulfanilamide from *p*NBSA was lost rapidly, while the ability to reduce 2,4-dinitrophenol was retained with the xanthine dehydrogenase activity during the early purification steps. 90 per cent of the activity of the original liver homogenate toward *p*NBSA was lost in the initial 56° heating step, while two-thirds of the activity toward 2,4-dinitrophenol was retained. Since the ability to reduce 2,4-dinitrophenol practically disappeared when this enzyme preparation was treated with Pangestin, while the xanthine dehydrogenase activity was retained, either an enzyme other

TABLE V

Comparative Reduction of 2,4-Dinitrophenol and p-Nitrobenzene Sulfonamide by Different Tissues with Added DPNH in 30 Minutes

Tissue	Species	2,4 Dinitrophenol	<i>p</i> NBSA
		Reduced per gm tissue	Sulfanilamide formed per gm tissue
Liver	Rat	298	450
	Chicken	326	420
	Pigeon	24	344
Kidney	Beef	15	104
Heart	Rat	116	121
	Beef	68	122

than xanthine dehydrogenase was responsible for the reduction of 2,4-dinitrophenol or the activities of the native xanthine dehydrogenase were altered by this digestion step

Aspergillus niger—The reduction of *p*-nitrobenzene sulfonamide to sulfanilamide by a preparation of *A. niger* was carried out anaerobically at 37.5° for 30 minutes under the conditions previously described for liver homogenates. *A. niger* was grown as surface cultures on liquid media for 4 to 6 days at 30°. Before sporulation, the mycelial felt was harvested, blotted, frozen for 3 hours, and homogenized with 3 times its weight of cold 0.1 M K_2HPO_4 . For the preparation of acetone powders, the frozen tissue was homogenized with 10 times its weight of cold acetone in a Waring blender, washed with acetone and peroxide-free ether, and dried under a vacuum. For assay, the acetone powders were homogenized with 20 ml of cold 0.1 M K_2HPO_4 per gm of powder. At least 90 per cent of the original

activity of the acetone powder was retained for 1 month when stored in the freezer

A. niger was grown on three different media (a) the KNO_3 medium of Nicholas (18), (b) the same medium but containing 4 gm per liter of NH_4NO_3 in place of the 5 gm of KNO_3 , and (c) the same as (b) but also containing 10 γ per ml of tungsten (as Na_2WO_4). Since the media were not especially purified to remove Mo, all three culture solutions contained adequate molybdenum for maximal growth on a nitrate medium (19). The amount of W in (c) was sufficient to prevent growth on a KNO_3 medium, and it decreased the growth by about one-half on the NH_4NO_3 medium. Growth was less abundant on KNO_3 , and 6 days were required to produce a weight comparable to that obtained on the W-free NH_4NO_3 culture in 4 days.

When the mold was grown on a KNO_3 medium, 4 ml of a 1:4 mycelial homogenate formed the equivalent of 33 γ of sulfanilamide per gm of fresh mycelium per 30 minutes. Supplementation with 0.06 mg of FAD or 3.2 mg of TPNH gave 43 and 89 γ of sulfanilamide, respectively, while the two together yielded 163 γ . DPNH was only 50 to 60 per cent effective as TPNH, FMN was 80 per cent as effective as an equal weight of FAD in the system. With FAD- and TPNH-supplemented systems, the mycelial homogenate prepared from the mold grown on the NH_4NO_3 medium gave 222 γ of sulfanilamide, tungstate added to the NH_4NO_3 medium did not suppress this reaction, since such a mycelial homogenate formed 258 γ of sulfanilamide. When the NH_4NO_3 medium was freed of Mo (18), the activity of the FMN plus the TPNH-supplemented mat was unchanged (219 γ of sulfanilamide), the addition of 2 γ of W (as Na_2WO_4) per 60 ml of the Mo-free NH_4NO_3 growth medium (20) was also without a major effect, since the mat formed 196 γ of sulfanilamide. There was no nitro-reducing activity in the filtrates or any of the culture media.

Acetone powders of the mycelial mat grown on an unpurified NH_4NO_3 medium formed approximately 120 γ of sulfanilamide per 30 minutes per gm of fresh tissue (190 mg of dry powder) when supplemented with FMN alone. Added DPNH did not stimulate this activity appreciably but restored about 50 per cent of the original value when added to a dialyzed preparation containing FMN. Table VI shows the effect of adding various substances to the unsupplemented acetone powder homogenate. Ferrous ions stimulated the reaction and overcame the inhibition by cyanide or Versene. 8-Hydroxyquinoline was without effect in the presence or absence of ferrous ions. Cysteine and glutathione stimulated the reaction with or without ferrous ions. Manganous ions were without effect, while

Zn^{++} and Cu^{++} inhibited the activity approximately 50 and 100 per cent, respectively. About 75 per cent of the activity of the acetone powder could be extracted with 0.1 M K_2HPO_4 .

Since no inhibition was caused by the absence of Mo, with or without W in the growth medium, it seems clear that the reduction of *p*NBSA to sulfanilamide by *A. niger* does not involve a Mo-containing enzyme and is therefore different from nitrate reductase. The stimulation by ferrous ions and the utilization of the pyridine nucleotides as substrates suggest an iron-flavoprotein enzyme as the active mediator of this reaction.

TABLE VI
*Effect of Ferrous Ions and Other Substances on Activity of A. niger
Acetone Powder in Reduction of pNBSA to Sulfanilamide*

Additions		+Fe ⁺⁺
None	90	123
8-Hydroxyquinoline	95	123
KCN	57	130
Versene (ethylenediaminetetraacetate)	60	201
Cysteine	139	228
Glutathione	144	160

Each Thunberg vessel contained 4 ml. of a 1:21 acetone powder homogenate plus 1 mg. of *p*NBSA in a total volume of 5.6 ml. 0.02 mmole of each listed substance was added, except that 0.2 ml. of saturated 8-hydroxyquinoline was used, and the Fe^{++} was increased to 0.04 mmole in the KCN and Versene experiments. The results are recorded as micrograms of sulfanilamide formed per 190 mg. of acetone powder (\approx 1 gm. of fresh mycelium) per 30 minutes.

DISCUSSION

It is evident from these results that a number of enzymes are capable of reducing organic nitro groups, and that different systems exhibit some specificity toward different nitro substrates. While rat liver xanthine oxidase is one of the enzymes capable of reducing *p*NBSA, it does not appear to be primarily responsible for this reaction in the liver homogenate. Enzymes located in both the soluble supernatant and particulate fractions which utilize the reduced pyridine nucleotides as substrates seem to be of greater importance in this reduction. Xanthine oxidase can also use the pyridine nucleotides and acetaldehyde as substrates, but this enzyme is not present in the particulate fraction and cannot be responsible for all of the activities with these substrates in the dialyzed homogenate or supernatant fraction. The various activities in rat liver toward *p*NBSA were all affected to about the same degree by tungstate feeding and are presumably

Mo-containing enzymes However, Mo is not required for the reduction of organic nitro groups since the enzyme in *A. niger* is independent of Mo and W

Depleting the rat liver enzymes by tungstate feeding had much less effect on the reduction of *p*NBSA *in vivo* than *in vitro* This result was similar to that of previous experiments in which xanthine oxidase was removed almost completely from the liver by a low protein diet (21) or by tungstate feeding (8), without any effect being evident on the uric acid and allantoin excretion in the intact rat

SUMMARY

Feeding Na_2WO_4 (72 mg per kilo of diet) to rats for 3 to 4 weeks to remove xanthine oxidase and other molybdenum-containing enzymes from the tissues decreased the ability of the liver homogenate to reduce *p*-nitrobenzene sulfonamide (*p*NBSA) by 80 per cent or more, this defect was prevented by including 50 mg of Na_2MoO_4 per kilo in the diet A protein-free diet also removed xanthine oxidase from the liver and markedly decreased its ability to reduce *p*NBSA

After an oral dose of *p*NBSA, 80 per cent of the urinary metabolites were in the reduced form when the diet was adequate, but only 62 to 66 per cent was reduced when the diet contained tungstate Tungstate feeding did not interfere with the acetylation of sulfanilamide when the latter was administered as such

Milk xanthine oxidase was inactive in the reduction of *p*NBSA and did not restore the activity *in vitro* of a Mo-depleted liver homogenate A crude rat liver extract containing xanthine oxidase reduced *p*NBSA with acetaldehyde, hypoxanthine, and reduced diphosphopyridine nucleotide (DPNH) or triphosphopyridine nucleotide (TPNH) substrates, but the activity of this enzyme was too small to account for the reducing ability of the original homogenate

Centrifugation of a normal rat liver homogenate at 40,000 r p m for 30 minutes yielded a precipitate fraction which utilized DPNH and TPNH in the reduction of *p*NBSA, but was relatively inactive with hypoxanthine, acetaldehyde, or succinate substrates The supernatant fraction was stimulated by DPNH, TPNH, and acetaldehyde but not by hypoxanthine or succinate, and similar results were obtained when the whole liver homogenate was dialyzed before testing Tungstate feeding did not elicit a differential response in these tests, since all activities were decreased proportionately

80 per cent of the activity toward *p*NBSA in pigeon liver was present in the high speed sedimented particulate fraction, and DPNH, TPNH, or acetaldehyde could be utilized as electron donor substrates An enzyme

prepared from the insoluble sedimented fraction of chicken liver was relatively specific for DPNH. The soluble supernatant fraction of beef liver contained three-fourths of the total activity and utilized DPNH, acetaldehyde, and, to a lesser degree, hypoxanthine substrates.

Rat and chicken livers reduced both *p*NBSA and 2,4-dinitrophenol satisfactorily, but pigeon and beef livers were much more effective with *p*NBSA than with 2,4-dinitrophenol. In the purification of chicken liver xanthine dehydrogenase, the ability of the preparation to form sulfanilamide from *p*NBSA was lost in the initial heating to 56°, whereas the ability to reduce 2,4-dinitrophenol was not lost until digested with Pangestin.

The reduction of *p*NBSA by *Aspergillus niger* was independent of Mo or W in the growth medium. Ferrous ions stimulated the reaction and overcame the inhibition by cyanide or Versene, reduced pyridine nucleotides were utilized as substrates.

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N-FORMYLCYSTEINE SYNTHESIS IN MITOCHONDRIA FROM FORMALDEHYDE AND L-CYSTEINE VIA THIAZOLIDINECARBOXYLIC ACID*

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In the course of the studies in this laboratory on 1-carbon compounds, it was found that the accumulation of formaldehyde in the oxidative demethylation of sarcosine by mitochondria was eliminated in the presence of L-cysteine (1). When cysteine alone was added to the mitochondria, it was oxidized with an oxygen uptake of 1 μ atom per μ mole,¹ but only after a pronounced induction period (Fig. 1). Upon the addition of an equimolar quantity of formaldehyde to this system, the induction period was abolished and there was a rapid uptake of oxygen. However, as shown in Fig. 1, the total oxygen consumption did not exceed the level obtained with cysteine alone. Nevertheless, the added formaldehyde disappeared completely (1). When the molar ratio between formaldehyde and L-cysteine was varied, the oxygen consumption was determined by the level of cysteine (Table I) until enough was added to saturate the cysteine oxidase system. Then the addition of formaldehyde did increase the oxygen consumption by 1 μ atom per μ mole of formaldehyde.

These results suggested that cysteine was being converted through a reaction with formaldehyde into a new and more active substrate. Of the known cysteine and formaldehyde reaction products, thiazolidinecarboxylic acid appeared to fit best the quantitative results shown in Table I. This compound, which was discovered independently by Schubert (2) and Ratner and Clarke (3) 20 years ago, is formed at room temperature by the insertion of the carbon of formaldehyde between the S and N in a cysteine molecule to produce a saturated ring. At pH 5 and above, closure of the ring is extremely rapid (3). In view of these considerations, L-thiazolidine-4-carboxylic acid was synthesized and incubated with mitochondria. As shown in Fig. 1, the rate of oxygen consumption was slightly faster than

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¹ Despite the oxygen uptake, which is twice that required to oxidize the cysteine to cystine, paper chromatography of the reaction mixture gives only a single ninhydrin spot with the R_F of cystine.

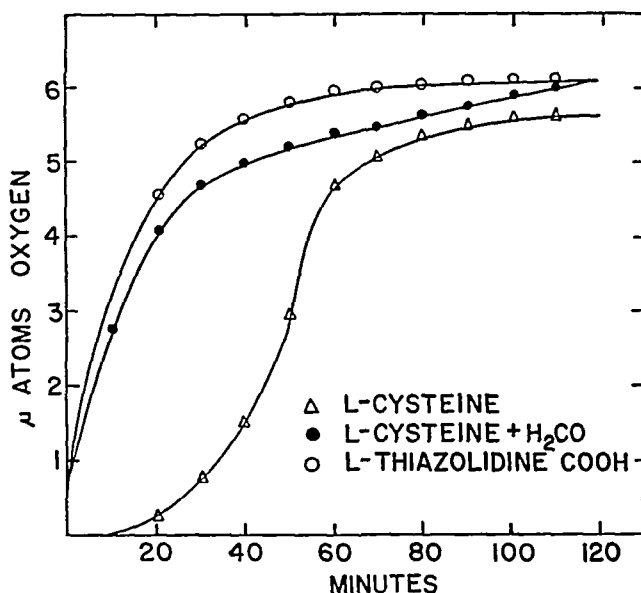


FIG 1 6 μ moles of each substrate were incubated with the mitochondria from 0.5 gm of liver in 2.2 ml of 0.075 M potassium phosphate buffer at pH 7.8. Formaldehyde alone is not oxidized by mitochondria.

TABLE I

Oxidation of Formaldehyde and L-Cysteine by Mitochondria

Mitochondria prepared from 0.5 gm of rat liver were washed and suspended in 2 ml of 0.075 M potassium phosphate buffer at pH 7.8. Substrates were added in 0.2 ml of buffer. The incubation was carried out for 2 hours at 37° in air.

Substrates		O ₂ consumption*	CH ₂ O recovered†
CH ₂ O	Cysteine		
μ moles	μ moles	μ moles	μ moles
5.3	0	0	4.5
0	1	1.0	0
5.3	1	0.9	3.7
0	3	3.0	0
5.3	3	3.1	1.5
0	6	6.2	0
5.3	6	6.2	0
0	12	7.1	0
0	16.5	11.5	0
5.3	16.5	16.9	0

* Corrected for the low endogenous value of approximately 6 μ l

† Formaldehyde contained in the trichloroacetic acid filtrate of the reaction mixture. An additional 0.8 μ mole of formaldehyde was bound by the trichloroacetic acid precipitate.

that produced by the consecutive addition of cysteine and formaldehyde. Moreover, the total oxygen uptake was the same in both cases (1). Finally, when the mitochondrial oxidation of cysteine plus formaldehyde was stopped by heating the reaction mixture, a compound was found which possessed the R_F of thiazolidinecarboxylic acid. All of these observations indicated that the metabolism of cysteine and formaldehyde in mitochondria proceeded via this compound.

Chemical Properties of Metabolite—The reaction mixture resulting from the mitochondrial oxidation of L-thiazolidinecarboxylic acid, or L-cysteine plus formaldehyde, was analyzed for a variety of functional groups. Ta-

TABLE II
*Analysis for Functional Groups of Substrates and
of Mitochondrial Incubation Mixture*

The supernatant fraction was prepared by heating the incubation mixture at 90° for 10 minutes and then centrifuging it

	—NH ₂ *	—SH	Formaldehyde		Formate
			Free	Periodate†	
CH ₂ O	—	—	+	—	—
Cysteine	+	+	—	—	—
Thiazolidine-COOH	+	—	—	+	—
Supernatant fraction‡	—	+	—	—	—
Hydrolyzed supernatant‡	+	+	—	—	+

* —NH₂ determined by ninhydrin method

† The formaldehyde produced by treating the preparation with periodate

‡ The same analytical results were obtained whether the substrate was formaldehyde plus cysteine or thiazolidinecarboxylic acid

ble II summarizes the most instructive findings, together with the results of the same tests on the related substrates which are included for comparison. The metabolism of cysteine and formaldehyde was accompanied by the complete disappearance of free amino groups and formaldehyde. Furthermore, formaldehyde did not appear when the supernatant fraction was treated with periodate, a procedure which liberates formaldehyde from thiazolidinecarboxylic acid. When the substrate was thiazolidinecarboxylic acid, its oxidation was accompanied by the generation of sulfhydryl groups. When the substrates were cysteine and formaldehyde, the sulfhydryl groups first disappeared and then, as the oxidation proceeded, reappeared. There was no loss of carboxyl groups during the incubation, as was shown by potentiometric titration of the reaction mixture. Acidifying and heating the supernatant fraction produced a compound which

possessed free amino groups. Its R_F was the same as that of cystine, or cysteine, treated in a similar fashion. When the acid-hydrolyzed supernatant fraction was distilled, the distillate was found to contain formic acid.

A single compound which possesses all of the qualities displayed by the incubation mixture is *N*-formyl cysteine. The observed oxygen uptakes (Fig. 1) agreed with the bivalent dehydrogenation required for the conversion of cysteine plus formaldehyde, or of thiazolidinecarboxylic acid, to this product.

Isolation of Radioactive N,N'-Di-formylcystine from Oxidized Reaction Mixture—Since the sulfhydryl groups in the reaction mixture disappeared slowly upon standing,² it was decided to oxidize them to the disulfide before attempting the isolation of the metabolite. In such experiments, both radioformaldehyde plus cysteine and radiothiazolidinecarboxylic acid were incubated with the mitochondria. After the oxygen uptake had ceased, oxygen was bubbled through the supernatant fraction in the presence of cupric ions until sulfhydryl groups could no longer be detected. *N,N'*-Di-formylcystine was then isolated by the carrier technique. As shown in Table III, it contained approximately 80 per cent³ of the incubated C^{14} , irrespective of whether this had been added as radioformaldehyde or as radiothiazolidinecarboxylic acid. Radioactive carbon dioxide and radioformaldehyde could not be detected as reaction products.

Evidence that the radioactivity in the carrier was due to *N,N'*-di-formylcystine, and not to contamination with some other radioactive metabolite, is presented in Table IV. Recrystallization from water and from methanol did not lower the specific activity. When the isolated material was hydrolyzed with acid, the cystine produced was devoid of radioactivity, all being found in the sodium formate isolated from the hydrolysate.

Identification of Metabolite As N-Formylcystine—From the evidence presented thus far, it could not be decided with certainty whether all, or only a small part, of the metabolic product was *N*-formylcystine, rather than *N,N'*-di-formylcystine. Indeed, the possibility could not be excluded that the metabolite was some labile precursor, for example, the dehydrogenated thiazolidinecarboxylic acid, which was converted to these compounds during the carrier isolation. To resolve these questions, *L*-thiazolidinecarboxylic acid was incubated with mitochondria, as soon as the

² There was no apparent diminution in the intensity of the nitroprusside reaction 2 hours after the supernatant fraction of the incubation mixture had been prepared. However, it was definitely less after storage in the refrigerator overnight and negative at the end of 48 hours.

³ In view of the ease with which the formyl group may be hydrolyzed in acid solution, it is likely that some loss occurred when the proteins in the incubation mixture were precipitated with trichloroacetic acid.

oxygen uptake had ceased, *N*-ethyl maleimide was added to the incubation mixture to trap any sulfhydryl compounds present (4) From the chro-

TABLE III

Metabolism of L-Radiothiazolidinecarboxylic Acid and Its Precursors, Radioformaldehyde and L-Cysteine, by Mitochondria

6 μ moles of each substrate were incubated Both radioactive compounds contained 1.8×10^4 c p m per μ mole, corrected for self-absorption The experimental conditions were the same as those given in Table I

Experiment No	Substrate	Oxygen consumption	Isolated <i>N,N'</i> -difor mylcystine*
		<i>microatoms</i>	<i>per cent incubated C¹⁴</i>
1	C ¹⁴ H ₂ O + cysteine	6.2	78
	Radiothiazolidine-COOH	6.1	80
	Radiothiazolidine-COOH†	0	3
2	C ¹⁴ H ₂ O + cysteine	5.8	77
	Radiothiazolidine-COOH	5.9	84
	C ¹⁴ H ₂ O + cysteine†	0.5	1

* Isolated by the carrier technique after converting the sulfhydryl groups in the reaction mixture to the disulfide

† Incubated with mitochondria previously heated for 10 minutes in a water bath at 90°

TABLE IV

*Specific Activity and Distribution of C¹⁴ in Isolated *N,N'*-Di-formylcystine*

The compounds are listed in the order of their preparation

Compound	C p m per mmole
<i>N,N'</i> -Di-formylcystine*	4.4×10^4
Recrystallized from water	4.7×10^4
“ “ methanol	4.7×10^4
Cystine	0
Sodium formate†	2.35×10^4

* The *N,N'*-di-formylcystine, isolated from the incubation of radiothiazolidine-carboxylic acid and radioformaldehyde plus cysteine (Table III), was pooled and mixed with an equal weight of carrier

† Since each molecule of *N,N'*-di-formylcystine yields 2 molecules of formic acid upon hydrolysis, the specific activity per millimole was reduced by one-half

matographic analysis in Fig. 2, it will be seen that only one spot was obtained and that this spot possessed the R_F of the *N*-ethyl maleimide derivative of *N*-formylcystine Furthermore, in the absence of maleimide a single spot was again found, this time with the R_F of free *N*-formylcystine Aeration of the incubation mixture before chromatography resulted in the

disappearance of *N*-formylcysteine and the appearance of *N,N'*-diformylcysteine. It can be concluded from these experiments that the primary end product of the metabolism of L-thiazolidinecarboxylic acid by liver mitochondria is *N*-formylcysteine.

Enzyme Specificity—The potency and specificity of the L-thiazolidinecarboxylic acid dehydrogenase of mitochondria are shown in Table V

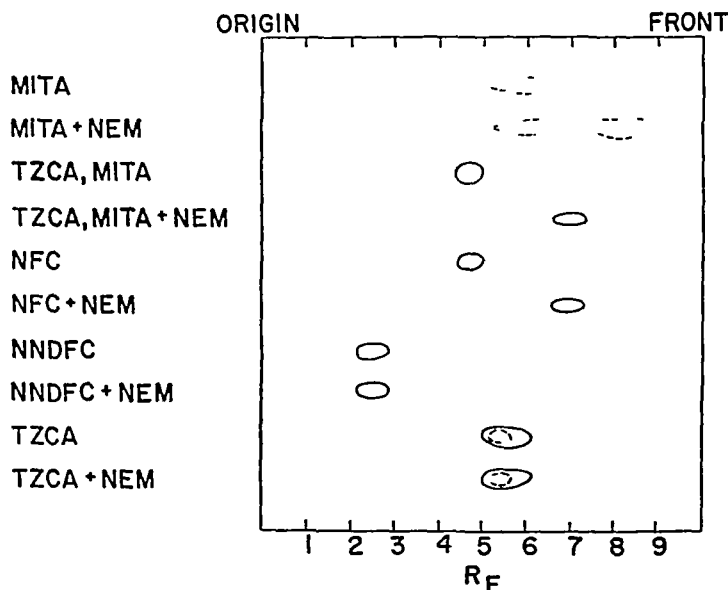


FIG 2 Chromatogram of the product of thiazolidinecarboxylic acid metabolism in mitochondria. 12 μ moles of substrate were incubated for 2 hours with the mitochondria from 0.5 gm of liver in a volume of 2.2 ml of buffer. 40 μ l of the supernatant fraction were spotted on Whatman paper No. 1 and developed in an ascending 80 per cent phenol-water system. The standards were dissolved in the endogenous supernatant fraction. Solid lines indicate platonic iodide spots, and broken lines indicate ninhydrin spots. The faint ninhydrin spots obtained with the endogenous material (shown at the top) have been omitted elsewhere for the sake of clarity. The following abbreviations have been used, MITA = mitochondria, NEM = *N*-ethyl maleimide, TZCA = L-thiazolidinecarboxylic acid, NFC = *N*-formylcysteine, NNDFC = *N,N'*-diformylcysteine. The *N*-ethyl maleimide was added to the whole incubation mixture as soon as the oxygen consumption had ceased.

6 μ moles of this substrate saturated the mitochondria isolated from 0.5 gm of liver. Under these conditions the initial rate of oxygen consumption ranged from 3 to 4 μ l per minute, depending on the preparation. The enzyme appears to be highly specific for the thiazolidine ring, the introduction of a methylene carbon to give the next higher homologue, L-1,3-thiazane-4-carboxylic acid, resulted in the complete loss of substrate activity (5). Likewise, removal of the carboxyl group from the ring to give 1,3-thiazolidine entirely abolished substrate activity. Substitution

of a methyl or propyl radical for one of the hydrogen atoms at position 2 reduced the total oxygen consumption by approximately 75 per cent (Table V). Whether even this low oxygen consumption was due to L-thiazolidinecarboxylic acid dehydrogenase is questionable, since the product of the reaction was not an acyl cysteine but free cysteine.

Djenkolic acid (6) is not oxidized by the mitochondria. Neither is *N*-formylcysteine or *N,N'*-diformylcystine metabolized by these particles. Apparently the mitochondrial cysteine oxidase requires a free amino group for its activity. Consequently, sulfhydryl groups, added to mito-

TABLE V

Oxidation of L-Thiazolidinecarboxylic Acid and Related Compounds by Mitochondria

The conditions of the experiment are given in Table I. Oxygen consumptions are the averages obtained from several experiments performed in triplicate.

Substrate	Quantity	Oxygen consumption
	μmoles	microatoms
L-Thiazolidine-COOH	6	6.4
"	10	10.0
"	12	11.8
"	18	15.3*
L-1,3-Thiazane-COOH	10	0
Thiazolidine	10	0
2-Methylthiazolidine-COOH†	10	2.4
2-Propylthiazolidine-COOH†	10	2.4
<i>N</i> -Formyl-L-cysteine	6	0
<i>N,N'</i> -Diformyl-L-cystine	3	0
Djenkolic acid	5	0

* After 3 hours, the oxygen consumption was 18 microatoms.

† From unpublished experiments of H. J. DeBey and C. G. Mackenzie.

chondrial preparations as *N*-formylcysteine, endure much longer than if added as free cysteine.

The supernatant fraction of liver which remains after the removal of mitochondria oxidizes thiazolidinecarboxylic acid either not at all or at a very slow rate. However, when the compound is incubated with the whole homogenate, the 2 hour oxygen uptake is increased by 1.5 μatoms for each micromole of added thiazolidinecarboxylic acid.

Pathway of Formaldehyde and Cysteine Metabolism in Mitochondria—In view of the experiments reported here, together with the studies of Ratner and Clarke (3) on the synthesis of thiazolidinecarboxylic acid, the metabolism of formaldehyde and L-cysteine by liver mitochondria may be formulated as shown in Fig. 3. Formaldehyde, which is hydrated in solution (7), condenses rapidly with the sulfhydryl group of cysteine to form the

S-methylol compound (3) This compound immediately undergoes ring closure to yield *L*-thiazolidinecarboxylic acid (2, 3) As indicated in Fig 1, the oxidation of *L*-cysteine by the cysteine oxidase of mitochondria is not fast enough to compete effectively with the conversion of cysteine to thiazolidinecarboxylic acid

The *L*-thiazolidinecarboxylic acid is attacked by a specific enzyme, the ultimate effect of which is to add 1 atom of oxygen to the compound On the basis of our present knowledge of biological oxidations, it seems likely

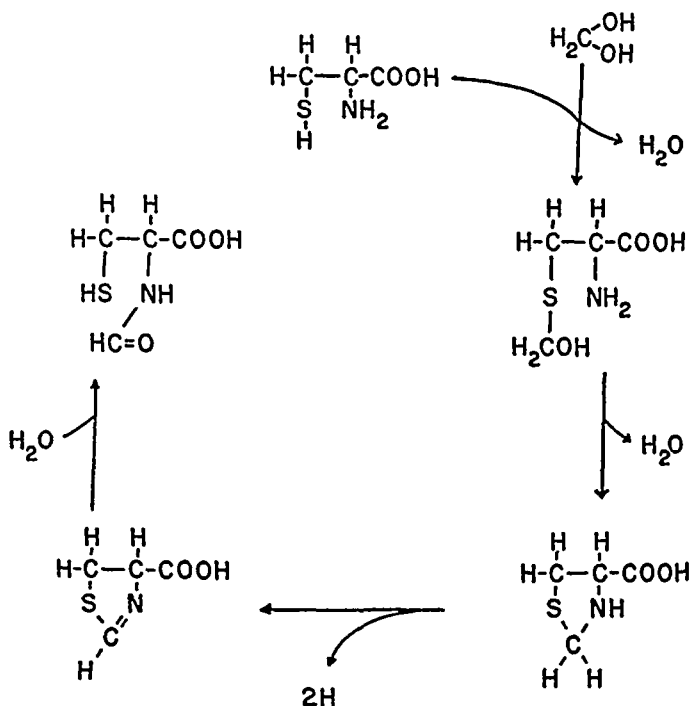


FIG 3 Conversion of formaldehyde and *L*-cysteine in mitochondria to *N*-formylcysteine via thiazolidinecarboxylic acid

that this is accomplished by a divalent dehydrogenation to yield the corresponding thiazolinecarboxylic acid, followed by the hydration of the double bond and migration of an H atom from O to S to open the ring and produce *N*-formylcysteine Whether either of the latter reactions is enzymatic is not presently known

Taken as a whole, this series of reactions represents the transfer of the aldehyde group from S to N with its concomitant dehydrogenation to produce the acid The oxidation takes place without any permanent change in the oxidation level of the cysteine, yet, in a sense, the cysteine has activated the aldehyde with respect to an enzymatic attack in the absence of added coenzymes Thus, these reactions are a model for the oxidation of

an aldehyde, whether the S and N which form the covalent bonds with it are both on a protein, both on a coenzyme, or are divided between the two. In this particular instance, the L-cysteine resembles a coenzyme which possesses both the S and N. Indeed, the cysteine would function as a coenzyme in the oxidation of formaldehyde if it could be regenerated by the hydrolysis of *N*-formylcysteine.

This hydrolytic reaction appears to occur in the living animal, for we have found that L-thiazolidinecarboxylic acid can replace L-cystine as a supplement to a low methionine diet. Furthermore, L-thiazolidinecarboxylic acid, on the basis of its sulfur content, is just as active a source of cystine for growth as is L-cystine itself.⁴

Finally, another aspect of thiazolidinecarboxylic acid metabolism should be mentioned. This odorless crystalline compound represents "frozen" sulfhydryl groups which may be liberated inside the cell as a consequence of enzymatic activity. Since these frozen sulfhydryl groups are not subject to oxidation to the disulfide form during digestion, absorption, and transport, it follows that in detoxification reactions thiazolidinecarboxylic acid could be more effective than cysteine. This possibility has been investigated, in collaboration with Dr Julia B. Mackenzie, by comparing the potency of thiazolidinecarboxylic acid with that of cysteine in preventing the massive pleural effusion and death produced in adult rats by thiourea (8). It was found that L-thiazolidinecarboxylic acid was approximately 5 times more active in this respect than cysteine (9). These results indicate the possibilities of a new sulfhydryl therapy based on the administration of metabolically generated sulfhydryl groups.

EXPERIMENTAL

Mitochondria—A modification of the procedure of Schneider and Hogeboom (10) was employed in which the mitochondria were isolated at a relatively low centrifugal force. Chopped liver was suspended in 0.25 M sucrose, in a ratio of 1 gm. per 1 ml., and the cells were broken in a glass homogenizer in a cold room at 1°. The homogenate was strained through doubled cheesecloth and the volume was measured. 1 ml. was considered to contain 0.5 gm. of liver. 0.25 M sucrose was added to increase the volume 5-fold, and the nuclei and unbroken cells were removed by centrifuging at 0° and 1700 r.p.m. for 5 minutes in an International refrigerated centrifuge with a No. 822 head. This speed corresponded to a force at the center of the centrifuge tubes of $420 \times g$. The supernatant fraction was decanted and centrifuged at $2300 \times g$ (4000 r.p.m.) for 20 minutes, a procedure which removes most of the mitochondria. Although centrifugation for

⁴ Unpublished data, H. J. DeBey and C. G. Mackenzie.

an additional 20 minutes will precipitate 10 to 20 per cent of the large granules that remain in suspension, in order to save time these were decanted along with the buffy layer. The isolated granules, which are spherical in shape and stain with Janus green, were suspended in 0.075 M potassium phosphate buffer at pH 7.8. The buffer was introduced slowly at first and with constant stirring to prevent agglutination of the particles. 4 ml of buffer were added for each gm of liver. The mitochondria were then centrifuged for 3 minutes at $1330 \times g$ (3200 r p m), the supernatant fraction was removed by decanting, and an equal volume of fresh buffer was added. This washing procedure was repeated two more times, and the mitochondria were finally suspended in 4 ml of buffer per gm of liver.

Incubation Procedures—2 ml of the washed mitochondria were added to each Warburg flask without the addition of cofactors. Substrates were added in 0.2 ml of buffer at pH 7.8. Cysteine and formaldehyde were placed in opposite side arms and the cysteine was tipped in first. The center wells of the flasks contained 0.2 ml of 20 per cent KOH.

After the oxygen uptake had ceased, the contents of each Warburg flask were transferred to a centrifuge tube together with 2 ml of wash water. When chromatograms were to be run, the proteins were precipitated by immersing the tubes in a boiling water bath for 10 minutes, otherwise they were removed by the addition of an equal volume of 10 per cent trichloroacetic acid.

Chromatography—Ascending chromatograms were run on Whatman No. 1 paper in an 80 per cent phenol and water system. Free amino compounds were detected by spraying with ninhydrin, and S compounds were located by dipping the paper in a solution of platinum iodide according to Toennies and Kolb (11), after first extracting the paper with acetone-ether to remove phenol. The latter test was also applied to papers previously treated with ninhydrin, thus permitting the location of S and amino compounds on the same paper.

A hydrolysate of the protein-free reaction mixture was prepared for chromatography by adding an equal volume of 1 N HCl and heating in a boiling water bath for 30 minutes. The solution was then evaporated to dryness at the water pump, resuspended in water, and again dried to remove excess HCl. This procedure hydrolyzed the *N*-formyl bond, but did not degrade thiazolidinecarboxylic acid, cysteine, or cystine.

Analytical Procedures—Formaldehyde was measured in the trichloroacetic acid filtrate of the reaction mixture, both before and after periodate oxidation, by the procedure of Frisell, Meech, and Mackenzie (12). Formic acid was determined in the distillate obtained from the acidified incubation mixture by the method of Benedict and Harrop (13). The nitroprusside test for $-SH$ was carried out in a solution of $NaHCO_3$. Negative tests were always confirmed with 2 N NaOH as the solvent.

Radioformaldehyde and radiocarbon dioxide were determined by methods described earlier (14). Sulfhydryl compounds were trapped in the incubation mixture by the addition of a 0.5 per cent solution of *N*-ethyl maleimide (4). 1.5 μ moles of reagent were added per micromole of S compound incubated.

Substrates—The L-cysteine and the djenkolic acid were obtained from the Nutritional Biochemicals Corporation. The formaldehyde was Mallinckrodt, analytical reagent formaldehyde solution.

L-Thiazolidine-4-carboxylic acid was prepared by a modification of the method of Ratner and Clarke (3). 4.13 mmoles of L-cysteine were dissolved in 3 ml of air-free water and 0.31 ml of the formaldehyde solution mentioned above, containing 4.13 mmoles, was added with stirring. The solution, which became slightly warm, was allowed to cool at room temperature. Crystals of thiazolidinecarboxylic acid appeared within 5 minutes to 2 hours in different preparations. 1 ml of cold 95 per cent alcohol was added and the preparation was placed in the refrigerator. The next morning the crystals were filtered and dried at the water pump. The compound was recrystallized twice from boiling water and dried at the pump with alcohol and ether. The compound contained 10.5 per cent N and melted with decomposition at 198–199°, uncorrected.

Thiazolidinecarboxylic acid, labeled in the 2 position with C¹⁴, was synthesized from radioformaldehyde (Isotopes Specialties Company, Inc.).

L-1,3-Thiazane-4-carboxylic acid was synthesized by the procedure described by Wriston and Mackenzie (5).

Thiazolidine hydrochloride was synthesized from mercaptoethylamine hydrochloride (Evans Chemetics, Inc.) by the procedure of Ratner and Clarke (3). It melted at 180°, uncorrected.

N,N'-Di-formyl-L-cystine was synthesized by the procedure of du Vigneaud, Dorfmann, and Loring (15). The N analysis agreed with the calculated value.⁵ The compound melted at 192°, uncorrected.

N-Formyl-L-cysteine was synthesized by suspending 1.2 gm of L-cysteine in 10 ml of formic acid and adding 5 ml of a 1:1 mixture of formic acid and acetic anhydride. After the cysteine had dissolved, the solution was allowed to stand for 15 minutes and several volumes of 1:1 benzene and ligroin were added. The precipitate was filtered and dried at the pump. It gave a strong nitroprusside test and a negative ninhydrin test. The N content was 10.1 per cent of the calculated value.⁵ The compound melted with effervescence at 131°.

Isolation and Hydrolysis of Radioactive N,N'-Di-formylcystine—The contents of each Warburg vessel obtained from the isotope experiments were transferred to centrifuge tubes together with two 1 ml portions of wash

⁵ We are indebted to Dr. Julia B. Mackenzie for the N analysis.

water 10 mg of non-isotopic *N,N'*-diformylcystine were dissolved in each tube and the proteins were precipitated by the addition of 1.4 ml of 20 per cent trichloroacetic acid. The tubes were centrifuged for 10 minutes and the supernatant fraction was decanted into plastic centrifuge tubes. The solutions were then titrated with 2 *N* NaOH to the end point of phenolphthalein. These operations were performed as rapidly as possible to minimize the hydrolysis of the formyl group. 0.5 ml of 0.02 *M* CuCl₂ was added to each tube and oxygen was bubbled through the solutions until the nitroprusside reaction was negative. This required about 1.5 hours.

The solutions were heated to approximately 60° with hot water and 300 mg of carrier *N,N'*-diformylcystine were dissolved in each tube. The tubes were placed in the refrigerator and on the next day the supernatant fluid was removed by decanting. The *N,N'*-diformylcystine was washed twice with ice-cold water and was then suspended in absolute alcohol and transferred to a planchet. After the granules had settled out, the alcohol was drawn off with a pipette and the precipitate was dried under an infrared lamp and weighed. These and all subsequent samples were counted with a thin mica window Geiger-Muller counter and corrected for self-absorption. The counts were calculated in terms of total carrier employed and the yield was expressed as a per cent of the incubated C¹⁴.

The radioactive *N,N'*-diformylcystine was recrystallized from water and dried at the pump with cold absolute ethanol and ether. The melting point was 194°, uncorrected. 260 mg of the recrystallized compound were dissolved in approximately 7 ml of boiling absolute methanol under a "cold finger" and the solution was placed in the refrigerator. The crystals were dried at the pump with absolute ethanol and ether.

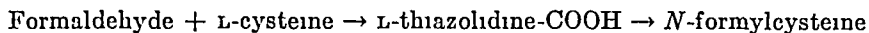
180 mg of this material were diluted to 1 gm with non-isotopic *N,N'*-diformylcystine and dissolved in 70 ml of 0.5 *M* H₂SO₄. The solution was refluxed for 30 minutes and 250 ml of doubly distilled water were added. The diluted solution was then distilled at 90 mm of Hg and 25° until a volume of approximately 20 ml remained. The distillate was collected in a flask immersed in an ice and brine bath. The residual solution in the distillation flask was brought to pH 5 with 2 *N* NaOH. The resulting precipitate was washed with cold water, alcohol, and ether, and was dried. The compound became nitroprusside-positive after cyanide treatment and possessed the *R_f* of cystine.

The distillate was titrated with carbonate-free 2.5 *N* NaOH to pH 6 by using glass electrodes. This solution was concentrated to 25 ml at the water pump and then lyophilized. The salt obtained melted at 255°, uncorrected. Admixture with sodium formate did not depress the melting

point. The activity of the sodium formate was multiplied by 1000 and then divided by 180 to correct for the dilution referred to above.

SUMMARY

L-Thiazolidinecarboxylic acid is metabolized by liver mitochondria to produce a high yield of *N*-formylcysteine. This reaction is catalyzed by a specific thiazolidinecarboxylic acid dehydrogenase. When L-cysteine and formaldehyde are added to mitochondria, *N*-formylcysteine is also formed as follows:



Isotopic experiments have shown that the carbon of formaldehyde and the carbon in the 2 position of thiazolidinecarboxylic acid are the sources of the formate carbon.

This series of reactions is discussed as a model for the enzymatic oxidation of aldehydes and as a means of producing sulfhydryl groups within the body for detoxication or other purposes.

The assistance of Mrs. Margaret M. Cooper during the early phases of this work, of Mrs. Marcia M. Michelson during the chromatographic studies, and of Mrs. Clelio J. Bates in preparing the figures is gratefully acknowledged.

Addendum—Since this article was submitted, Cavallini and coworkers (16) have reported the formation of *N,N'*-diformylcystine from thiazolidinecarboxylic acid and a liver enzyme preparation. In their paper, they refer to our earlier work as follows, "Recently Mackenzie and Harris and Mackenzie studied the fate of the portion of the molecule formed from formaldehyde. We have devoted our attention to the metabolic fate of the substrate as a whole." We should like to point out that the abstract by Harris and Mackenzie (17) was equally concerned with the cysteine and formaldehyde parts of the molecule, and was entitled "Metabolism of formaldehyde and cysteine via thiazolidinecarboxylic acid." Moreover, we concluded, "This compound (thiazolidinecarboxylic acid) is then dehydrogenated by a specific oxidase to yield the corresponding thiazoline, which in turn is hydrolyzed to *N*-formylcysteine."

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FAT METABOLISM IN HIGHER PLANTS

IX ENZYMIC SYNTHESIS OF LONG CHAIN FATTY ACIDS BY AVOCADO PARTICLES*

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The discovery and isolation of the multienzyme system responsible for β oxidation of fatty acids to acetyl CoA¹ have stimulated investigation of the synthesis of long chain fatty acids by multiple β condensation of the acetyl CoA. In 1953 Van Baalen and Gurin (1) were the first to demonstrate that acetate was incorporated into fatty acids by aqueous extracts of pigeon liver. In 1953 Stansly and Beinert (2), employing the purified enzymes of the β -oxidative system, showed the synthesis of butyryl CoA from acetyl CoA. In 1955 Hele and Popják (3) described soluble enzyme systems from rat and rabbit mammary glands which catalyze the synthesis of long chain fatty acids from acetate in the presence of ATP, CoA, and DPN. Langdon (4) has made the important observation that TPNH is required for the incorporation of acetate-C¹⁴ into the higher fatty acids by soluble liver systems. Recently Gibson and Jacob (5) and Wakil, Porter, and Tietz (6), using three different protein fractions obtained from pigeon liver and a mixture of cofactors and substrates, showed the synthesis of long chain fatty acids from acetate.

In higher plants, Newcomb and Stumpf (7) observed that slices of cotyledons of both germinating and developing peanuts had the capacity to incorporate acetate-C¹⁴ into long chain fatty acids. Gipple and Kurtz (8) demonstrated that acetate-1-C¹⁴ was incorporated by developing flax fruits into long chain fatty acids which were labeled predominantly in the odd-numbered carbon atoms. Sisakyan and Smirnov (9) supplied acetate-C¹⁴ to sunflower chloroplasts and isolated long chain fatty acids with relatively low radioactivity. No cofactor requirements were demonstrated.

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¹ The following contractions are employed: ATP, adenosine triphosphate, CoA, coenzyme A, DPN, diphosphopyridine nucleotide, TPN, triphosphopyridine nucleotide, Tris, tris(hydroxymethyl)aminomethane, UTP, uridine triphosphate, GTP, guanosine triphosphate, CTP, cytosine triphosphate, ITP, inosine triphosphate, TCA, trichloroacetic acid.

This paper presents evidence that a cell-free particulate system, isolated from avocado mesocarp, catalyzes the synthesis of long chain fatty acids from acetate in the presence of ATP, CoA, and Mn^{++} under anaerobic conditions

Methods and Materials

Preparation of Enzyme System—Avocado fruits (*Persea americana*) were purchased as needed from local food stores. The Fuerte variety was used in the majority of experiments. Generally one fruit, the mesocarp of which weighed about 200 gm, supplied sufficient particles for one set of experiments.

The fruit was peeled, the seed removed, and the remaining fleshy mesocarp was treated in the cold room (3–4°) as follows. The mesocarp was passed through a food grater and ground with acid-washed sand in a chilled mortar (10) with 1 volume of cold 0.4 M sucrose-0.2 M Tris buffer at pH 7.3. The homogenate was squeezed through four layers of cheesecloth, and the filtrate was centrifuged in a Servall superspeed angle centrifuge type SS-1 for 7 minutes at $500 \times g$ to remove fine debris. The supernatant fluid was then centrifuged at $10,000 \times g$ for 30 minutes, and the sediment was washed by resuspension in 50 ml of sucrose buffer and by centrifugation again at $10,000 \times g$ for 30 minutes. The final sediment was suspended in an appropriately small volume of sucrose-Tris buffer (containing about 30 mg of protein per ml) and used within a few minutes.

Procedure—Enzyme reactions were carried out in small Pyrex tubes containing the particulate suspension, cofactors, and substrate labeled with C^{14} . The tubes were flushed with nitrogen, stoppered, and shaken gently on a rotary shaker for 2 hours at 30°. After incubation, the contents of each tube were added to an equal volume of saturated $KHSO_4$ solution in a Maizel-Gerson tube. The lipides were extracted once from each tube with 1 ml of chloroform. The chloroform extracts were evaporated at low heat on to aluminum planchets, and the fatty residues were counted directly with a thin window Geiger-Muller tube, assuming infinite thinness. Activity was expressed as the per cent of incorporation, counts per minute in chloroform extract $\times 100$ divided by counts per minute in substrate. Repeated control experiments showed that unincorporated acetate-2- C^{14} was completely lost when the chloroform extract was evaporated on the aluminum planchets.

Formation of Hydroxamic Acid Derivatives—Hydroxylamine with low salt content was prepared by adjusting a concentrated solution of hydroxylamine sulfate with saturated $Ba(OH)_2$ to pH 6.5 and precipitating the $BaSO_4$. Water was then added to yield a molar solution of hydroxylamine. Hydroxylamine was added to reaction mixtures at various stages in their

incubation, and the hydroxamic acid derivatives were isolated by the method of Stadtman and Barker (11). Colorimetric analyses for hydroxamic acid derivatives formed from non-radioactive substrates were carried out by the method of Lipmann and Tuttle (12).

Identification of C^{14} -Labeled Lipides—Portions of the chloroform extract were fractionated on MgO-Celite and CaCO_3 -Celite columns, as described by Strain (13). The radioactivity of each fraction was determined by counting the eluates on aluminum planchets.

Other portions of the chloroform-soluble lipide material were saponified by being refluxed in 10 per cent ethanolic KOH for 5 hours. Fatty acids were separated from the acidified saponification mixtures by steam distillation. Long chain fatty acids tended to collect in the condenser and were washed out with a few ml of ethyl ether. The aqueous solutions were then made slightly alkaline and reduced to small volumes in a flash evaporator, model FE-2 (Laboratory Glass Supply Company), at 40° . The concentrates were acidified with 6 N H_2SO_4 and extracted with ethyl ether. The ether was evaporated to dryness, and the residues were redissolved in minimal volumes of petroleum ether. The fatty acids were chromatographed by the reversed phase method of Kaufmann and Nitsch (14). In this method, Whatman No. 3 paper is immersed in kerosene for 15 minutes and then dried in a well ventilated hood for 4 hours at room temperature. Fatty acids are applied as free acids dissolved in hexane, and the paper was developed in a descending direction with a 9:1 acetic acid-water solvent system at room temperature. The fatty acids and their R_F values are as follows: capric 0.60, lauric 0.50, myristic 0.39, palmitic 0.23, oleic 0.24, and stearic 0.15.

With short chain fatty acids, a portion of the above ethyl ether extract was treated with concentrated NH_4OH solution to form the ammonium salts of the fatty acids which were chromatographed by the method of Kennedy and Barker (15).

Radioactive spots were located by exposure of the papers to x-ray film for from 5 to 10 days, depending upon the activity of the material chromatographed. In addition, long chain fatty acids were located by treating the paper with a 1 per cent lead acetate solution, washing it with water, and exposing the lead salts of the fatty acids to H_2S . Fatty acids appeared as dark spots of lead sulfide. This method is sensitive to 0.1 μmole quantities of fatty acid (16).

Unsaturated fatty acids were demonstrated by the change in their chromatographic movement after reduction by hydrogen with a platinum catalyst in a pressure reaction apparatus (Parr Instrument Company).

Aliquots of fatty acids, synthesized by the avocado particulate system from carboxyl- and methyl-labeled acetate, were decarboxylated by the

Schmidt azide reaction by the method of Phares (17) The CO_2 released was collected in 20 per cent KOH, precipitated with barium acetate, and counted as BaCO_3

Reagents—Carboxyl- and methyl-labeled acetate- C^{14} , both with a specific activity of 1.0 mc per mmole, were purchased from TraceLab, Inc., Boston. CoA and ATP were purchased from the Pabst Brewing Company and the Sigma Chemical Company, and reduced glutathione from the Nutritional Biochemicals Corporation.

RESULTS AND DISCUSSION

Properties of Enzyme System—A study of various centrifugal fractions of the avocado mesocarp homogenate showed that the particles most active in incorporating acetate into the lipid fraction were sedimented in 30 minutes at $10,000 \times g$. When examined under the light microscope, this fraction appeared to consist mainly of particles at the limit of optical resolution in addition to a small number of chloroplasts. When the mesocarp was divided into the green outer and the yellow inner sections, the green tissue contained far more chloroplasts than the yellow, but, according to weight, particles prepared from each section gave about equal incorporation of acetate. Since the procedure used to prepare the particles was identical with that of Millerd *et al.* for isolating avocado mitochondria (17), the particles comprising the enzyme system in question appear to be "mitochondria" in the generally accepted sense of that term. Addition of supernatant solution to mitochondrial preparations caused no stimulation, but instead inhibition of acetate incorporation. This is in contrast to the work of Gibson and Jacob (5) and of Hele and Popják (3). The former found a requirement for both soluble and particulate fractions of pigeon liver for fatty acid biosynthesis, whereas the latter demonstrated all activity to be in the soluble portion of rat and rabbit mammary gland homogenates.

The enzyme system in avocado mitochondria is quite labile. Heating at 50° for 5 minutes at pH 7.0 destroyed its activity completely, freezing overnight at -20° reduced the activity 80 per cent, merely allowing the preparation to stand at 0° for 2 hours lowered its ability to incorporate acetate into lipids by 50 per cent.

Anaerobic incubation of the reaction mixtures resulted in greater incorporation of acetate than did aerobic incubation. However, the difference between the two was not as large as was expected, i.e. incubation in Warburg vessels exposed to the air showed 9 per cent incorporation against 15 per cent in tubes flushed with nitrogen. In other experiments, reactions carried out either in Warburg vessels flushed with nitrogen and containing O₂ sorbent (Burrell Corporation) in the center well or in Thunberg tubes

flushed carefully with nitrogen gave no greater acetate incorporation than did reactions in small tubes flushed with nitrogen and closed with rubber stoppers

A time study of the reaction system showed that after 90 minutes there was no further acetate incorporation. The mixtures were routinely incubated for 2 hours.

Cofactor Requirements—Cofactor requirements for the mitochondrial system are shown in Table I. The requirements for ATP, CoA, and Mn^{++} are clearly evident. The effect of ATP is particularly striking, although a rather high concentration was required for optimal activation. This

TABLE I

Cofactor Requirements for Incorporation of Acetate-2- C^{14} into Lipides by Particulate System of Avocado Fruit

The complete reaction mixture contained 1.0 ml of mitochondrial suspension (about 15 mg of protein) in 0.4 M sucrose, 0.2 M Tris buffer, ATP 5.5×10^{-3} M, CoA 3×10^{-4} M, glutathione 2.7×10^{-3} M, $MnSO_4$ 5×10^{-4} M, NaF 2.7×10^{-3} M, acetate-2- C^{14} 5×10^{-5} M, 0.05 μ c, 35,000 c.p.m., phosphate buffer 5.5×10^{-3} M. Total volume in each tube, 1.8 ml. The tubes were flushed with nitrogen and incubated for 2 hours at 30°.

Components	Per cent substrate acetate 2 C^{14} incorporated into lipides
Complete system	27.5
Without ATP	0.1
“ CoA	4.1
“ $MnSO_4$	1.4
“ glutathione	25.2
“ NaF	22.5

suggested a contaminating nucleotide other than ATP as the active nucleotide. As a source of such a nucleotide, a complex of mononucleotides, isolated by charcoal adsorption from acid-extracted yeast cells,² was added to ATP. No stimulation was observed. Other nucleotides such as UTP, GTP, CTP, and ITP were ineffective. The specific requirement for Mn^{++} is also rather remarkable. Although different metal ions were tested for activity, either alone or in combination with Mn^{++} , none of them stimulated acetate incorporation significantly. Substrate concentration was also critical. Increases in concentration of acetate over about 2.0×10^{-4} M caused inhibition of the incorporating system. Inhibition of a mitochondrial system by short chain fatty acids was also observed in the β oxidation of butyrate by mitochondria of germinating peanut cotyledons (18).

² The authors are indebted to Dr. Rao Sanadi for this preparation.

It should be pointed out that throughout the course of these experiments there was considerable variation from day to day in the activity of the particulate preparations. This was undoubtedly related to the condition of the avocado fruit and its history of handling and storage. Nonetheless, although activity varied quantitatively (1 to 30 per cent incorporation of acetate), no instance was ever found of qualitative difference in cofactor requirements.

A marked requirement for a reducing system could not be shown although the addition to the reaction mixtures of DPN and TPN with α -keto glutaric acid gave a slight but consistent stimulation of acetate incorporation. DPN and the reducing system, ethanol dehydrogenase and ethanol,

TABLE II

Effect of CoA and ATP on Formation of Acetyl Hydroxamates by Avocado Particulate System

The reaction systems were as in Table I except for the addition of 0.5 mmole of hydroxylamine, pH 6.5, and 0.2 μ mole of acetate-2- C^{14} , 54,000 c.p.m. The hydroxamic acids were chromatographed, detected, and eluted as described in the text. In each case only one spot, corresponding to the acetyl hydroxamate, appeared on the chromatogram.

Reaction mixture	Radioactivity of eluted material
	c.p.m.
Complete	842
Without CoA	138
“ “ ATP	0
“ ATP	0

alone or in combination with TPN and its reducing system, glucose-6-phosphate dehydrogenase and glucose 6-phosphate, produced no stimulation. Moreover, DPNH alone had no effect on the incorporation of acetate. This lack of effect by DPN and TPN reduced outside the mitochondrion may be related to a permeability barrier into the particle.

Fatty Acid Activation—To demonstrate the enzymes of fatty acid activation, the mitochondrial system was incubated with hydroxylamine and either labeled or non-labeled fatty acid substrates. Acyl CoA derivatives will form hydroxamates which can be detected by their production of a colored complex with $FeCl_3$ and by their chromatographic movement. In experiments with labeled acetate, the reaction mixtures were chromatographed on paper with water-saturated butanol, and the spots were located on x-ray film, eluted with ethanol, and counted (11). As indicated in Table II, only the acetyl hydroxamate was evident on the chromatograms. It is of

interest to point out that the condensation products of acetyl CoA, C₄, C₆, C₈, etc (CoA derivatives), did not accumulate in hydroxylamine-trapping experiments in which hydroxylamine was added at different time intervals. In other experiments with 5 μ moles of non-radioactive acetate, butyrate, and octanoate, colorimetric analysis gave evidence for the formation of acetyl, butyryl, and octanoyl hydroxamates. Palmitic and oleic acids gave no reaction (Table III). Tris buffer extracts of acetone powder of the fruit mesocarp also formed hydroxamic acid derivatives when incubated with cofactors and non-radioactive acetate, but the activity of the powder decreased rapidly with storage. From the above evidence it was concluded that avocado mitochondria possess a fatty acid-

TABLE III

Specificity of Fatty Acid-CoA-Activating System of Avocado Particles

The reaction systems were as in Table I except that 0.5 μ mole of non-labeled fatty acid substrate was substituted for acetate-2-C¹⁴ and 0.5 mmole of hydroxylamine, pH 6.5, was added at the beginning of the reaction. Quantitative estimations of hydroxamic acid derivatives are described in the text.

Substrate	Hydroxamate*
	<i>μmole</i>
Acetate	0.84
Butyrate	0.49
Octanoate	0.38
Palmitate	0.00
Oleate	0.00

* Succinyl hydroxamate as standard

activating system which in our preparations is able to activate acetate, butyrate, and octanoate in a decreasing order of activity. These results are similar to those in plant extracts (19) and in bacteria and animals (20).

Identification of C¹⁴-Labeled Reaction Products—Since chloroform extracts obtained from mitochondrial mixtures were green in color, it was decided to determine first whether any of the activity was present in pigmented compounds. Portions of the labeled chloroform-soluble materials were fractionated on CaCO₃-Celite and MgO-Celite columns by us and by Dr Chichester, Department of Food Technology, University of California, Davis. Activity was found to be associated with the esterified xanthophyll fraction in all cases. However, upon elution and saponification, the colored fraction lost its activity. This radioactivity was then recovered from the colorless aqueous extracts of the saponified eluate. It was concluded from this evidence that the fatty acids synthesized by the reaction mixtures were esterified in part with the naturally occurring xanthophylls. This

conclusion is tentative since it is possible that triglycerides are associated with esterified xanthophylls on the chromatographic columns. Experiments in which the particulate enzymes were incubated with cofactors, non-radioactive acetate, and glycerol-1-C¹⁴ indicated that glycerol could serve as an acceptor of fatty acids. The question of whether the final occurrence of the newly synthesized fatty acid is as a xanthophyll ester, a triglyceride, or both, requires further investigation.

For preliminary identification of the fatty acids synthesized by the particulate preparations, the reversed phase paper chromatography procedure developed by Kaufmann and Nitsch (14) was employed. When the saponified lipide was chromatographed, two radioactive spots were observed by exposure of the chromatogram to x-ray film. One spot (R_F 0.22) was larger and considerably more radioactive than the other (R_F 0.14). These R_F values correspond to the positions of palmitic or oleic and stearic acids, respectively. When an aliquot of the saponified material was hydrogenated and chromatographed, the 0.22 area decreased while the 0.14 area increased in size and radioactivity. This would indicate the accumulation of stearic acid which originated from the reduction of oleic acid present in the saponified lipide material. All the radioactivity of newly isolated lipide material, when chromatographed by the Kaufmann and Nitsch (14) system, remained at the origin. This indicated the absence of free fatty acid and the occurrence of the radioactive acids as neutral fats.

A more precise analysis was made by separating the saponified acids by gas-liquid partition chromatography. We are greatly indebted to Dr. K. P. Dimick of the Western Utilization Research Laboratory, Albany, California, for his cooperation in this phase of the work. Enzymically prepared radioactive fatty acids were esterified to the methyl esters by treatment with diazomethane, and 2.5 mg. each of methyl laurate, methyl myristate, methyl palmitate, methyl stearate, and methyl oleate were added as carriers. The ester mixture dissolved in *n*-heptane was injected into the column heated to 214° (General Electric silicone supported in brick dust in a 5 foot column). As the peaks appeared on the Brown recorder, the samples were collected and assayed for radioactivity (Table IV). Although only 20 per cent of the total applied radioactivity was recovered, it can be clearly seen that higher fatty acids became labeled. It was not possible to separate stearic and oleic acids by this method, but because stearic acid is never labeled to any noticeable extent on the basis of reversed phase chromatography and does not occur in avocado fruits in significant amounts (21), it is concluded that the major radioactivity of the stearic-oleic fraction is contributed by oleic acid.

No labeled short chain fatty acids accumulate in reaction mixtures as evidenced by the Kennedy and Barker method (15). Water-soluble

compounds that were left after chloroform extraction were recovered by Soxhlet ether extraction. Upon chromatography with butanol-formic acid-water, the presence of labeled citric, malic, succinic, and probably α -ketoglutaric acids was observed. Since labeling in these TCA cycle acids of the tricarboxylic acid cycle was reduced considerably by rigorously anaerobic incubation of the reaction mixtures, it is assumed that acetate- C^{14} enters the TCA system through the presence of residual oxygen. Neither butyrate-1- C^{14} nor propionate-1- C^{14} was incorporated into chloroform-soluble compounds by the particulate system.

In view of these experiments it is concluded that palmitic and oleic acids are the only fatty acids synthesized in detectable quantity by the avocado mitochondrial system. Similar preliminary studies have been made with

TABLE IV

Gas-Liquid Chromatography of Enzymically Synthesized Fatty Acids

Radioactive fatty acids were isolated from the avocado particulate system and treated as described in the text

Fatty acid (methyl ester)*	Emergence time	Radioactivity
	min	c p m
Lauric	7.0	0
Myristic	14.2	18
Palmitic	28	280
Oleic†	55}	469
Stearic†	60}	

* 2.5 mg of the methyl esters of these fatty acids added as carriers

† Stearic and oleic acids were not separable. See the text

mitochondria isolated from cotyledons of germinating peanuts. Acetate-2- C^{14} was readily incorporated into the chloroform-extractable lipids when the mitochondria were incubated in the presence of ATP, CoA, and Mn^{++} . Without the cofactors no incorporation was observed.

The results of the Schmidt decarboxylation of fatty acids synthesized from carboxyl-labeled acetate are shown in Table V. Fatty acids were converted by catalytic reduction to the saturated fatty acids before decarboxylation to avoid oxidation at points of unsaturation by reagents of the Schmidt reaction. The results of the decarboxylation suggest strongly that the fatty acids are labeled along the whole chain. If the fatty acids are synthesized by the condensation of 2-carbon units, every other carbon in the chain should be labeled. Thus, in a 16-carbon fatty acid synthesized from carboxyl-labeled acetate the carboxyl group should contain one-eighth the total radioactivity in the molecule. The experimental results in Table V agree with this hypothesis.

The evidence of C^{14} distribution combined with the experiments demonstrating the presence of a conventional CoA-fatty acid-activating system supports the concept that fatty acid biosynthesis in the avocado system is through a mechanism essentially the reverse of β oxidation. This is the expected mechanism since β oxidation of fatty acids is known to occur in plants (18), and the condensation of acetyl CoA has been demonstrated to be the route of fatty acid biosynthesis in other organisms (20).

TABLE V

Schmidt Decarboxylation of Enzymically Synthesized Fatty Acids

Radioactive fatty acids were prepared and isolated from the avocado particulate system as described in the text. No attempt was made to separate the acids since only palmitic and oleic acids are the major ones. Control experiments with different concentrations of palmitic-1- C^{14} consistently gave 60 per cent yields. Therefore, a correction factor was applied to the radioactivity of CO_2 evolved. By assuming equal alternate labeling in the side chain of the C_{16} and C_{18} fatty acids and an average carbon chain length of 17, a factor of 1 to 8.5 was derived to relate the radioactivity in the terminal carbon (carboxyl) to that of the whole molecule. The result of decarboxylation of fatty acid enzymically synthesized from methyl labeled acetate- C^{14} is presented as a control.

Experiment No	Substrate enzymically incorporated into fatty acid	Total C^{14} in enzymically synthesized fatty acid	Corrected radioactivity of CO_2 evolved from carbon 1	Calculated radio activity in carbon 1 of enzymically synthesized fatty acid
		<i>c p m</i>	<i>c p m</i>	<i>c p m</i>
1	Acetate-1- C^{14}	1440	171	170
2	"	960	138	113
3	Acetate-2- C^{14}	1195	5	0

SUMMARY

Particles from avocado fruit mesocarp incorporate C^{14} -labeled acetic acid into esterified long chain fatty acids. Adenosine triphosphate, coenzyme A, and Mn^{++} are essential components of the system. Palmitic and oleic acids were the only labeled acids found to accumulate. Evidence is presented to support the theory that the mechanism of fatty acid formation in the avocado is by the condensation of coenzyme A-activated acetate units. The identity of the natural acceptor of fatty acids in the avocado mitochondrion is in doubt but may be a xanthophyll or a glycerol.

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MITOCHROME*

I ISOLATION AND PHYSICOCHEMICAL PROPERTIES

II ENZYMATIC EFFECTS

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Under controlled conditions, it is possible to isolate mitochondria with several of the known enzymes present in only partially active or completely inactive form (3-5). With these preparations a maximal efficiency of the system for oxidative phosphorylation can be approximated. When mitochondria are aged or damaged by exposure to hypotonic solutions, a dissociation of oxidation from phosphorylation is believed to occur so that, although oxygen uptake may continue at about the same rate, little or no esterification of inorganic phosphate is found (6-8). Hypotheses offered in explanation for these phenomena include the destruction of phosphorylation enzymes or coenzymes (9) and the activation of ATPase¹ and acid phosphatase with changes in the permeability of the mitochondrial membrane (3, 4).

Our studies, directed toward the restoration of the efficiency of phosphorylation in aged mitochondria, revealed a partial recovery of phosphorylation after the addition of serum albumin to the reaction mixture (Table I).

Since the recovery of phosphorylation with albumin was obtained with mitochondria aged for 76 hours at 0°, but failed when mitochondria aged for 70 minutes at 28° were used, the effect of albumin could be attributed to the reversal of a relatively simple change in mitochondria that occurred slowly at 0°. At room temperature, additional degradations occurred that were not reversed by albumin.

The nature of the albumin reactivation was revealed when it was found that a factor in the soluble proteins from aged mitochondria inhibited the aerobic phosphorylation of freshly prepared mitochondria and that the inhibition was counteracted by serum albumin (Table II).

* Preliminary reports of phases of the experimental work were made (1, 2).

¹ The following abbreviations are used: adenosine triphosphate = ATP, adenosine diphosphate = ADP, inorganic phosphate = P, protein-bound phosphate of undefined energy state = \neq P, Tris buffer = tris(hydroxymethyl)aminomethane.

These observations led to the isolation of the protein "uncoupling" factor designated as mitochrome, which is described in this report

TABLE I

Reactivation of Aerobic Phosphorylation of Aged Mitochondria by Serum Albumin

Time of incubation before reaction	Control		Albumin	
	Esterified P	Ratio, $\frac{P}{O}$	Esterified P	Ratio, $\frac{P}{O}$
Preparation A				
hrs at 0°	μ atoms		μ atoms	
4	18 2	2 0	19 0	2 0
27	11 5	1 7	13 5	2 0
49	11 2	1 5	14 4	2 0
72	7 2	1 1	13 0	1 9
76	7 1	1 4	11 7	2 0
Preparation B				
min at 28°				
0	11 9	1 8	11 7	1 8
15	11 8	1 8	11 3	1 8
30	4 4	1 1	8 0	1 7
46	1 3	0 5	5 4	1 4
70	0 0	0 0	0 0	0 0

Reactions were performed in Warburg flasks containing 2.6 ml of medium composed of 0.014 M phosphate buffer, pH 7.2, 0.02 M NaF, 0.009 M MgCl₂, 0.017 M KCl, 0.0017 M NaHCO₃, 0.003 M histidine at pH 7.2, 0.025 M α -ketoglutarate, pH 7.2, 0.011 M glucose, 7×10^{-4} M ATP, 160 units of yeast hexokinase prepared according to Berger *et al* to Step III (10), and 0.2 ml of mitochondria (1.2 mg of protein N) isolated from rat liver by differential centrifugation in isotonic sucrose (11). Reactivation was accomplished with 3.32 mg of commercial crystalline bovine serum albumin. Phosphate buffer, F⁻, and ATP were placed in the side arm, other components were added to the main compartment. After a 6 minute equilibration with air at 28°, the vessel contents were mixed and oxygen uptake was measured for 30 minutes. CO₂ was absorbed with 0.2 ml of 15 per cent KOH in the center well. Inorganic phosphate was determined by the method of Fiske and Subbarow (12) on an aliquot of the reaction mixture deproteinized with cold 15 per cent perchloric acid.

Isolation Procedure

Mitochrome was isolated first from the soluble proteins of aged mitochondria from rat liver. Apparently undefined changes that occur in mitochondria during the aging period are of critical importance for the successful isolation of a soluble mitochrome. This is emphasized by the additional

yields of soluble mitochondrion obtained from the insoluble fragments of extracted mitochondria after either partial digestion with trypsin or long term storage (3 to 6 months at -20°) in ethanolamine buffer at pH 9.0. Proteins similar to the rat mitochondrion were obtained from the liver tissue of rabbit, pig, cow, and monkey. Finally, it was found that mitochondrion could be isolated from whole homogenates of liver without a preliminary separation of the mitochondria. After the release or extraction of mitochondrion from the mitochondrial particle, the actual separation from other proteins is accomplished easily by virtue of the insolubility of mitochondrion

TABLE II
*Inhibition of Phosphorylation by Factor from Aged Mitochondria
and Reactivation by Albumin*

Addition to control reaction	Uptake		Ratio, $\frac{P}{O}$
	Oxygen	P	
	μatoms	μatoms	
None	11.3	20.3	1.8
Bovine serum albumin, 4 mg	11.2	26.6	2.4
Supernatant fraction from aged mitochondria	6.9	0	0
Supernatant fraction from aged mitochondria + albumin	11.1	18.7	1.7

Reactions were carried out with fresh mitochondria under conditions described in Table I, but with the indicated additions to the control reaction mixture. The supernatant fraction from aged mitochondria was prepared by centrifuging, at $25,000 \times g$, mitochondria that had been stored at 0° in isotonic sucrose until all phosphorylation activity was lost, 0.2 ml. of the clear supernatant solution (0.10 mg. of protein N) was used in the reaction.

in dilute solutions at pH 5, the isoelectric point, and by its insolubility in 0.25 saturated ammonium sulfate solution at pH 9.

Isolation from Mitochondria. Step I—Mitochondria prepared by differential centrifugation in isotonic sucrose solution (11) were stored as a 2 per cent suspension in isotonic sucrose at -20° until a stock of protein was accumulated. Approximately 50 gm. of the frozen stock were thawed and centrifuged at $20,000 \times g$. The sediment was homogenized in 0.1 M KCl-0.02 M NaHCO_3 solution or in 0.1 M ethanolamine buffer, pH 9.0, diluted to form a 2 per cent protein mixture, and aged for 4 days at 5° . The soluble protein was again separated by centrifugation at $20,000 \times g$ and the insoluble residue resuspended to form a 1 per cent protein suspension in the 0.1 M alkaline buffer. After being stirred overnight at 5° , the extracted proteins were separated by centrifugation at $20,000 \times g$, and the

clear supernatant solutions from the three extractions were combined. Approximately two-thirds of the total mitochondrial protein was soluble at this stage.

Step II—The combined extracts were adjusted to pH 4.5 with 1 M acetic acid and filtered through fluted paper in a cold room. The precipitate was homogenized in 0.1 M ethanolamine buffer, pH 9.1, diluted to a 0.5 per cent suspension, and filtered or centrifuged clear. With care, little denaturation takes place during the acid precipitation step, so that almost all of the precipitated protein obtained by the previous centrifugation can be dissolved in half the volume of ethanolamine buffer used in the first extraction. Any protein that remained insoluble in the ethanolamine buffer after this second extraction was discarded. The proteins that dissolved in the ethanolamine buffer were combined, adjusted to pH 5 with 1 N acetic acid, and the isoelectric precipitate that formed was removed by centrifugation ($1800 \times g$). After decantation of the clear, green supernatant solution, the precipitate was suspended in ethanolamine buffer, pH 9.1, and diluted to a 1 per cent solution. Any turbidity that remained after the solution was stirred for 2 hours at 5° was removed by centrifugation at $20,000 \times g$. This isoelectric precipitation step was repeated twice until the filtrate from the precipitated protein became colorless and contained less than 0.05 per cent protein. Approximately 10 per cent of the original mitochondrial protein was recovered at this step. Electrophoresis of the preparation at this stage showed that almost 85 per cent of the protein migrated as a single component with the mobility of mitochrome.

Step III—The last isoelectric precipitate was dissolved in 0.1 M ethanolamine buffer,² pH 9.5, and diluted to a protein concentration of 1 per cent. Solid sodium tetraborate was added to a final concentration of 0.05 M, and crystalline ammonium sulfate was added to 0.3 saturation. The precipitate was removed by filtration or centrifugation, redissolved in 0.1 M ethanolamine buffer,² and the salt fractionation was repeated at 0.3 saturation and finally at 0.25 saturation with ammonium sulfate in the presence of 0.05 M borate. The last precipitate was redissolved in sufficient ethanolamine

² Trace quantities of heavy metal precipitate mitochrome in a form that is difficult to redissolve and give the impression of denaturation. Use of ethylenediaminetetraacetate (Versene) prevents this to some extent. In some preparations, especially those from pig liver, the protein was associated with a lipid component from which it could not be easily separated. Although there was little difference in the enzymatic or electrophoretic properties of these turbid preparations, the absorbancy index at 280 and 260 $m\mu$ was significantly increased. When preparations of mitochrome did not dissolve to form a clear solution in the ethanolamine-Versene buffer, they were stored in alkaline buffer solution, pH 10, at -20° for a few weeks. Subsequent fractionation by isoelectric precipitation usually, but not always, resolved a clear soluble mitochrome preparation.

mine buffer to form a 0.8 per cent solution and dialyzed overnight against 10 times its volume of distilled H_2O at 5° . A slight residual turbidity was removed by centrifugation at $30,000 \times g$. The clear solution was then adjusted to pH 5.0 with 1 M acetic acid and the precipitate was removed by centrifugation and redissolved in 0.05 M Tris buffer, pH 8.0, for enzymatic studies. The yield of mitochrome ranged from 2 to 8 per cent of the original mitochondrial protein.

Mitochrome from Insoluble Mitochondrial Fragments after Digestion with Trypsin—The insoluble residue of mitochondria after ethanolamine extraction (6.1 gm) was again homogenized with 0.1 M ethanolamine buffer, pH 9.1 (0.7 per cent protein suspension), in a Waring blender with temperature maintained at 28° . Centrifugation at $1800 \times g$ for 15 minutes yielded a turbid suspension containing 2.4 gm of protein. Most of this protein (1.7 gm) was precipitated by centrifugation at $30,000 \times g$. This precipitate was homogenized in a Waring blender with 350 ml of 1 M ethanolamine-20 per cent glycerol solution, pH 10.5, at -5° . After 30 minutes at 0° , the suspension was centrifuged at $25,000 \times g$ for 15 minutes. The precipitate recovered (1.1 gm) was homogenized in 300 ml of 0.05 M ethanolamine buffer, pH 9.5, and, after the addition of 1 mg of crystalline trypsin, was incubated for 1 hour at room temperature. A partial clarification of the suspension occurred so that with centrifugation at $25,000 \times g$ a clear solution containing 750 mg of the extracted brown protein was obtained. Further fractionation of this solution according to the procedure outlined in Step III yielded 250 mg of mitochrome.

Mitochrome from Whole Homogenates of Liver—After the properties of mitochrome were established with preparations from mitochondria, the isolation procedure was reinvestigated to determine the feasibility of isolating the protein from whole homogenates of liver.

Whole beef or rat liver frozen and stored at -20° for several months was thawed and homogenized with 0.25 M sucrose-0.01 M Versene solution, pH 7, in a Waring blender to form a 2 per cent protein suspension. After centrifugation at $600 \times g$ to remove the nuclei and cells, the supernatant suspension was adjusted to pH 4.5 and the precipitate that formed was filtered overnight at 5° or centrifuged at $1800 \times g$. This precipitate, containing considerable denatured and insoluble protein, was homogenized in a Waring blender with 0.1 M ethanolamine buffer, pH 9.5 (1 per cent suspension), and stirred at room temperature for 2 hours. The suspension was then filtered through fluted paper in a cold room. To facilitate the extraction, a clogged filter with remaining protein was homogenized in a Waring blender after a volume of ethanolamine equivalent to that of the filtrate that had passed through was added, and the suspension of protein and paper pulp was poured on to a fresh filter. Filtration at this

point was very slow, requiring 3 to 4 days. During this time the filter paper was changed twice a day. The extraction of the precipitate was discontinued when the protein content of the filtrate dropped to 0.1 per cent. Mitochrome was isolated from the clear filtrate by isoelectric precipitation at pH 5.0 and by salt fractionation described previously in Steps II and III. In one preparation, starting with 2.5 kilos of whole beef liver, 23.6 gm of protein were obtained at Step I, 13.3 gm at Step II, and the final protein yield was 7.6 gm.

Frequently preparations of mitochrome from whole liver were obtained, associated with a deep red-colored protein which, when isolated in electrophoretically homogeneous form, gave the spectral characteristics of a cytochrome b_2 (absorption maxima at 424, 530, and 560 $m\mu$ after reduction with dithionite). This trace contaminant, amounting to almost 2 per cent of the total protein, was separated from mitochrome by chromatography. A 1 per cent solution of the protein in 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$ was passed through an adsorption column composed of equal parts of silica and Celite (13) that had been washed and equilibrated with the solvent. With the proportions of 10 mg of protein per gm of silica-Celite, all of the protein was adsorbed on the column. Approximately 60 per cent of the mitochrome, free from the red protein, was eluted from the column with 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$. The residual mitochrome and "cytochrome b_2 " were eluted with 1 M ethanolamine buffer, pH 9.

The mitochrome obtained from either mitochondria or whole homogenate of liver is a brown-colored protein that is soluble and stable in dilute alkaline buffers for a few weeks at 4°. It can be stored at -20° for at least a year with only minor losses of activity.

Physical and Chemical Properties

The properties of the isolated mitochrome summarized in Table III refer to the dry weight of the salt-free protein. Nitrogen was determined by the micro-Kjeldahl procedure described by Miller and Houghton (14). Phosphorus was determined after digestion of the protein with H_2SO_4 and H_2O_2 (12). The bond between phosphorus and protein was not split appreciably by hydrolysis in 1 N HCl for 15 minutes and was transformed quantitatively to inorganic P only after complete digestion of the protein. A modification of the *o*-phenanthroline reaction was used for the iron content after wet digestion of the protein (13).

Light Absorption—The shape of the absorption spectrum of mitochrome in the ultraviolet and visible regions and the changes produced by reduction with dithionite and interaction with CO are shown in Fig 1, A and B. The data, obtained with a Beckman recording spectrophotometer, are reported in terms of the absorbancy index, $a = ((\log I_0/I)/bc)$, where

TABLE III
Physicochemical Properties of Mitochrome Isolated from Beef Liver

Composition	
	<i>per cent</i>
Nitrogen	14.21
Phosphorus	0.28
Iron	0.055

Light Absorption

Wave length <i>mμ</i>	a_s^*
280	1.486
410	0.313
422 (reduced)	0.235
420 (" CO)	0.350

Electrophoretic Mobilities in 0.1 μ Buffers

Buffer	pH	U†
Tris	9.0	-5.80
"	8.4	-5.45
"	7.0	-4.77
Veronal	8.4	-5.40
Veronal-acetate	7.1	-4.80
"	6.4	-4.66
Acetate	3.8	+5.1
"	4.0	+4.5
Isoelectric point	5.1	0

Molecular Size

Sedimentation constant, $s_{20w} \times 10^{13}$	3.0
Diffusion constant, $D_{20w} \times 10^7$	3.1
Partial specific volume (V_1), ml per gm	0.78
Molecular weight	
M_s	107,000
M (analytical Fe)	102,000
M (light-scattering)	160,000
Dissymmetry constant, f/f_0	2.13
Viscosity increment hm ($\eta/\eta_0 - 1$)/ cV_1 $c \rightarrow 0$	18.3

* a_s = absorbancy index = absorbancy ($\log I_0/I$) per cm depth per 0.1 gm per cent

† U = mobility in sq cm per volt per second $\times 10^{-6}$

$\log I_0/I$ is the absorbancy (A), b the path length in centimeters, and c the concentration in gm per liter (15) Quantitative values for the absorption maxima that are analytically useful are given in Table III

Although the absorption spectrum of mitochrome showed the characteristic Soret band of a heme protein, the absorbancy of this band compared to that of the 280 $m\mu$ band was much less than is found with the more commonly known heme proteins This led to experimentation to determine whether the heme moiety was present as a contaminant All our attempts to separate the heme component without denaturing the protein were unsuccessful The assumption that the heme group was an integral part of the mitochrome molecule became more plausible with the

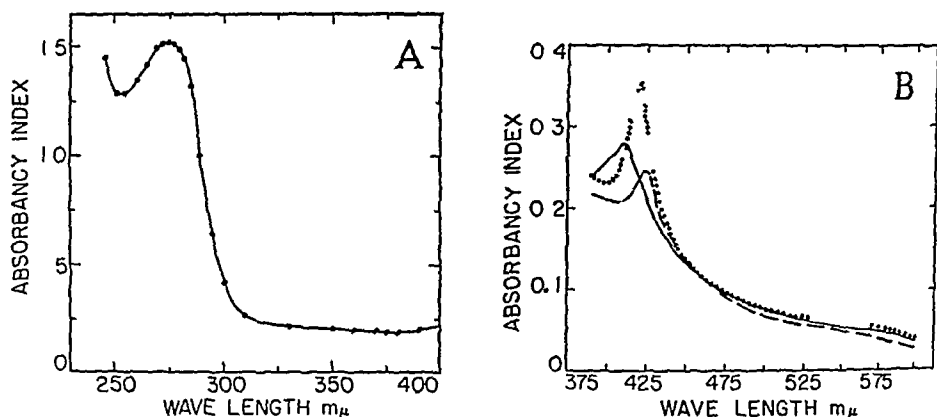


FIG 1 A, ultraviolet light absorption of mitochrome in 0.04 M phosphate buffer, pH 8.0 B, absorption of mitochrome (solid line), reduced mitochrome ($\text{Na}_2\text{S}_2\text{O}_4$) (broken line), and reduced CO mitochrome (dotted line) in 0.04 M phosphate, pH 8.0

quantitative agreement of the iron content of 0.055 per cent with the molecular weight obtained from sedimentation and diffusion data

Electrophoresis—Electrophoresis of mitochrome in 0.1 ionic strength buffers resolved a single boundary at pH ranges acid and alkaline to the isoelectric point of the protein Measurements of the mobility of mitochrome in 0.1 ionic strength Tris, Veronal, phosphate, and acetate buffers over the pH range 3.8 to 9.0 determined an interpolated isoelectric point at pH 5.1 (Table III) In the pH range 5 to 6 the low solubility of the protein precluded any reliable mobility calculation with the optical resolving power of the equipment used

A mixture of equal quantities of mitochrome and serum albumin migrated as a single component during electrophoresis in alkaline buffers (Fig 2) In buffers acid to the isoelectric point, the mixture was resolved into two components One component with a mobility of 2.6 showed the characteristic inhomogeneity of serum albumin at pH 4 first

described by Luetscher (16), the other component with mobility of 4.5 was identified as mitochrome (Fig 2, C). The electrophoretic indications of a protein-protein interaction between mitochrome and serum albumin were confirmed by sedimentation data. Ultracentrifugation of equimolar mixtures of mitochrome and serum albumin gave a monodisperse sedimentation pattern with an $s_{20,w} = 3.7$ for the mixture that was greater than $s_{20,w} = 3.0$ for mitochrome alone and less than $s_{20,w} = 4.4$ for albumin alone. The physicochemical evidence for interaction between mitochrome and serum albumin offers some reasonable explanation for the action of albumin in counteracting the enzymatic effects of mitochrome on intact mitochondria.

Molecular Weight—The molecular weight of mitochrome was obtained

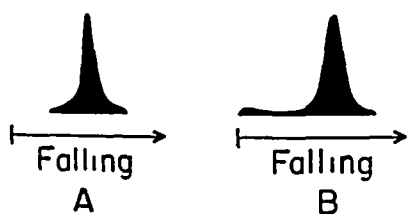


FIG 2

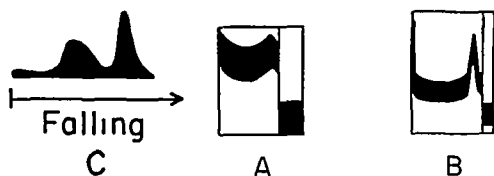


FIG 3

FIG 2 Electrophoretic patterns of the descending boundaries of *A*, mitochrome (pH 8.4, $\mu = 0.1$, U (mobility in sq cm per volt per second $\times 10^{-5}$) = -5.4), *B*, mitochrome + bovine serum albumin (1:1) (pH 6.8, $\mu = 0.1$, $U = -5.1$), *C*, mitochrome + albumin (1:1) (pH 4, $\mu = 0.1$, $U = 2.6, 4.5$)

FIG 3 Sedimentation patterns of *A*, 0.5 per cent mitochrome, *B*, a mixture of 0.58 per cent mitochrome and 0.42 per cent albumin in 0.1 M Veronal buffer, pH 9.1

from measurements of the sedimentation and diffusion constants of the protein. Values for the sedimentation constant were obtained by Dr M. MacKenzie with a Spinco ultracentrifuge. Solutions of a lyophilized preparation of mitochrome were made in 0.125 M phosphate buffer, pH 7.8, and in 0.1 M Veronal buffer, pH 9.1, with a final protein concentration of 1.0 and 0.5 per cent, respectively. Fig 3 shows representative sedimentation patterns of mitochrome and of a mixture containing 0.42 per cent serum albumin and 0.58 per cent mitochrome in Veronal buffer. In contrast to the electrophoretic homogeneity of mitochrome, the sedimentation pattern indicated a weight heterogeneity. This difference suggests either that the protein is an interacting aggregate of heterogeneous molecular species that migrates as a single component in an electric field or that the protein molecules are of the same species but that they associate to form a weight-heterogeneous molecular population. The monodisperse sedimentation pattern (Fig 3) obtained with approximately equimolar concentrations of mitochrome and albumin in the face of the polydisperse

pattern of the mitochrome alone intimates that mitochrome exists as an equilibrium mixture of unassociated and associated molecules of the same molecular species and that reaction with albumin displaces the equilibrium predominantly in the direction of an albumin-mitochrome compound

Diffusion measurements were made with the schlieren optical system of the Aminco-Stern electrophoresis apparatus on a 0.7 per cent solution of mitochrome in Veronal buffer, pH 9.1. Weight average diffusion constants $D_{20,w}$ were calculated by the method of moments and corrected to standard conditions as described by Lundgren and Waid (17).

With the Svedberg ultracentrifuge equation (17), a molecular weight of 107,000 was obtained from the data for the sedimentation constant, diffusion constant, and partial specific volume of mitochrome. This agrees with the minimal molecular weight of 102,000 calculated from the iron content of mitochrome with the assumption of 1 atom of iron per molecule (Table III). The molecular weight of mitochrome was also determined for us by Dr. R. Steiner who used a light-scattering method (18). The disagreement of the value of 160,000 with those obtained by the other methods might be attributed to the association of the mitochrome molecules that would produce a proportionately greater effect on the scattering of light.

The data summarized in Table III also permit the calculation of the frictional ratio or dissymmetry constant f/f_0 (17). The high value of 2.13 indicates that the molecule is not spherical. By making the usual assumptions that the molecule is a rigid, impenetrable, and unhydrated ellipsoid of revolution, the axial ratio of such an ellipsoid can be obtained from the frictional ratio or the intrinsic viscosity by using the graphical expression of the Perrin or the Simha relation (19). The intrinsic viscosity of the protein was obtained from specific viscosity measurements made with Ostwald-Cannon-Fenske type capillary viscosimeters (20). From an intrinsic viscosity of 18.3, the axial ratio for a prolate ellipsoidal model is 12.2. The axial ratio derived from the dissymmetry constant is 23.6. For an oblate ellipsoidal model, the axial ratios would be 25 from viscosity data and 35 from the frictional ratio. The differences found for the prolate model are much greater than has been reported generally for the two methods (21). Similar discrepancies, however, have been reported for the fibrinogen molecule (22).

Enzymatic Effects

Inhibition of Aerobic Phosphorylation by Mitochrome—The hypothesis that the diffusion of mitochrome from mitochondria caused an apparent inactivation of aerobic phosphorylation was confirmed by experiments exemplified in Table IV. In a system with freshly prepared mitochondria

demonstrating oxidative phosphorylation with nearly maximal efficiency, the phosphorus esterified via ATP to glucose with hexokinase was diminished progressively by increasing the concentration of mitochondria in the reaction mixture. In agreement with the thesis stated previously, serum albumin counteracted the inhibition so that phosphorylation was restored. This reversal of the mitochondria effect apparently was limited to mammalian serum albumins, since serum globulins, egg albumins, protamines, insulin, etc., were inactive.

Activation of Dephosphorylation of ATP by Mitochondria—Since mitochondria had no demonstrable effect on the isolated reaction transferring phosphate from ATP to glucose by yeast hexokinase, it seemed reasonable

TABLE IV
*Inhibition of Oxidative Phosphorylation by Mitochondria and
Reactivation by Serum Albumin*

Mitochondria added	Control		Albumin	
	Esterified P	Ratio, $\frac{P}{O}$	Esterified P	Ratio, $\frac{P}{O}$
mg	μatoms		μatoms	
0.0	25.7	2.7	24.6	2.9
0.29	18.1	2.4	22.5	2.9
0.57	11.2	1.2	22.0	2.7
1.14	1.3	0.3	14.7	2.3

Reactions were performed with fresh mitochondria under conditions described in Table I, but with the indicated additions of a purified mitochondria from rat liver to the reaction mixture. The reactivation experiments were carried out with 3.0 mg of crystalline bovine serum albumin in the reaction medium.

to attribute the effect of mitochondria to a reaction with sites on the surface of mitochondria. The result might be interpreted as a discharge of high energy P on the mitochondrial surface to inorganic P. In the absence of mitochondria, the isolated protein, mitochondria, had only a small and negligible action on ATP. However, a pronounced activation of ATP dephosphorylation was observed when mitochondria, that gave no indication of ATP hydrolysis, were added to the substrate in the presence of mitochondria. Since the inhibition of aerobic phosphorylation could be explained on the premise of a discharge of high energy phosphate on the mitochondria to inorganic phosphate, the more simple reaction of ATP dephosphorylation was investigated in more detail.

The activation of ATP dephosphorylation by mitochondria is illustrated in Fig. 4. During the first few minutes of the reaction, there is an apparent esterification of inorganic phosphate by the freshly prepared mito-

chondria With time and with increasing concentrations of the activator protein, the net effect is a hydrolysis of phosphate from ATP

The complex nature of the reaction with mitochrome was emphasized by the failure of first order and second order elementary theoretical kinetic expressions (23) to describe the data adequately Doubling the initial ATP concentration changed the order of the reaction from 0 to 3 A

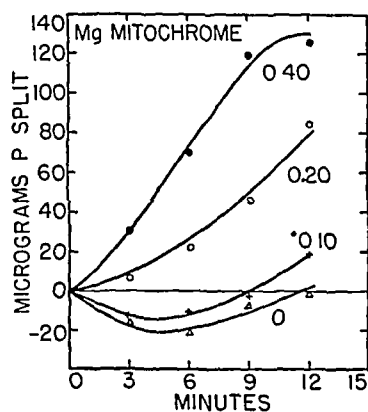


FIG 4

FIG 4 Phosphate split from ATP as a function of time with varied concentrations of mitochrome in the reaction mixture Reactions were carried out in air at 28° in 2.0 ml of solution with final concentrations of 0.025 M Tris buffer, pH 7.1, 1.25×10^{-3} M $MgCl_2$, 3.08×10^{-3} M ATP, 0.2 ml of mitochondria containing 0.554 mg of protein N, and the indicated quantities of mitochrome At stipulated times, the reaction was stopped by the addition of 1 ml of cold 15 per cent perchloric acid and the protein-free filtrate was analyzed for inorganic phosphate

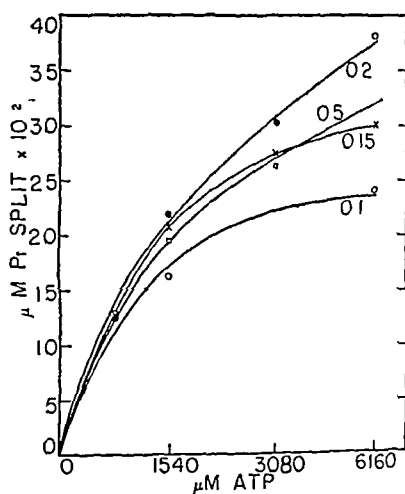


FIG 5

FIG 5 Effect of substrate concentration and mitochrome concentration on ATP dephosphorylation Reactions were carried out in 2 ml mixtures with final concentrations of 0.025 M Tris buffer, pH 7.1, 0.3 ml of mitochondria containing 1.27 mg of protein N in isotonic sucrose, and the indicated concentrations of ATP (micromolar) The mitochrome concentration (ml of 1.2 per cent solution) is labeled on each curve After 10 minutes incubation at 28° , the reaction was stopped by the addition of 1 ml of cold 15 per cent perchloric acid and analyzed for inorganic phosphate

4-fold increase over the initial substrate level produced a complicated reaction that followed no simple order With the reaction time fixed, it was evident empirically from the data presented in Fig 5 that the appearance of inorganic phosphate was an exponential function of the substrate concentration at any mitochrome concentration By plotting the data (Fig 5) as phosphate split *versus* mitochrome concentration at different substrate levels, it was apparent also that the activation effect of the mitochrome passed through a maximum and that the reaction was inhibited by higher concentrations of mitochrome

Albumin Effect—Since the inhibition of aerobic phosphorylation attributable to mitochondria was reversed by serum albumin, then the activation of ATP dephosphorylation produced by mitochondria should also be reversed by serum albumin if both inhibition of phosphorylation and activation of dephosphorylation are the result of the same action of mitochondria on mitochondria. This concept is supported by the data in Fig 6. As shown, the resultant dephosphorylation activity is dependent upon the concentration of mitochondria relative to the concentration of albumin.

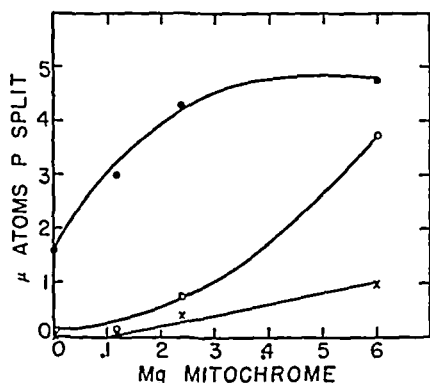


FIG 6

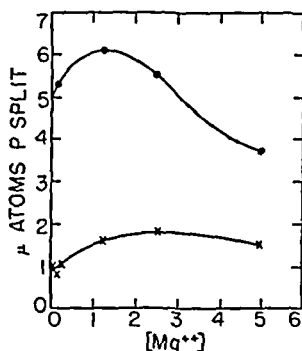


FIG 7

FIG 6 Effect of albumin on mitochondria activation of ATP dephosphorylation. Reactions in 2 ml of media containing 3.08×10^{-3} M ATP, 1.25×10^{-3} M MgCl_2 , 0.025 M Tris buffer, pH 7.2, 0.2 ml of mitochondria (0.84 mg of protein N), and the indicated quantities of mitochondria were run with no albumin (●), with 3.0 mg of albumin (○), and with 6.0 mg of albumin (×). After 10 minutes at 28° the phosphate hydrolyzed was determined as described in Fig 5.

FIG 7 Mg^{++} ion activation of ATP dephosphorylation by mitochondria in the presence (●) and absence (×) of mitochondria. Reactions were performed for 10 minutes under conditions described in Fig 5. $[\text{Mg}^{++}] = \text{M} \times 10^{-3}$.

With sufficient albumin the activation effect of mitochondria can be completely abolished.

The physical interaction of mitochondria and albumin demonstrated previously by electrophoretic and sedimentation experiments suggests the possibility that albumin exerts its action by masking functional groups on the protein or by a steric hindrance effect that blocks the complex from sites on the mitochondrial surface. The interaction of albumin and mitochondria is not dependent upon the albumin molecule *per se*, since partial denaturation with urea does not decrease its reaction with mitochondria. Methylation of the albumin molecule lowers its activity by approximately 50 per cent. Reaction with iodoacetic acid after urea denaturation, acetylation, or iodination completely abolishes the albumin effect. The iodinated albumin, in addition to being inactive with mitochondria, has an inhibitory action of its own on aerobic phosphorylation. It appears, then,

that the albumin effect with mitochrome is due to specific end groups in the peptide linkage of albumin and not primarily to the molecular configuration of the native protein

Mg⁺⁺ Activation—The effect of Mg⁺⁺ on the activation of ATP dephosphorylation is shown in Fig 7. In the absence of mitochrome, Mg⁺⁺ had only a small effect. In the presence of mitochrome, maximal activation was obtained with 1.25×10^{-3} M Mg⁺⁺, higher concentrations of Mg were inhibitory. The data indicate that the effect of mitochrome cannot be attributed primarily to a Mg⁺⁺ activation of ATPase induced by a trace metal content of the mitochrome.

Mechanism of Mitochrome Action

Over a limited substrate concentration range the kinetic data for mitochrome activation of ATP dephosphorylation followed the Lineweaver and Burk (24) formulation of the Michaelis-Menten equation to give a family of straight lines with divergent intercepts on the velocity axis and with different slopes. Although this might be interpreted as implicating mitochrome in the dephosphorylation of ATP without any direct interaction between ATP and mitochrome, *i.e.* non-coupling activation, the data *per se* offered no clearly defined mechanism for the reaction. This information was obtained by more direct procedures with the use of radioactive phosphate and tracer techniques.

Discharge of Mitochondrial Bound P³² by Mitochrome—A preparation of mitochondria containing bound radioactive phosphate (mitochondria \equiv P³²) was made by mixing 0.25 ml of P³² (20×10^6 c.p.m. per ml) with 4 ml of mitochondria (16 mg of protein N) in isotonic sucrose at 0°. Excess P³² was removed by centrifuging at $10,000 \times g$ and resuspending the mitochondria in cold isotonic sucrose three times. The last sediment was resuspended in 4 ml of 0.25 M sucrose. The control (Table V, Experiment A) was composed of 0.5 ml of mitochondria \equiv P³² and 3 mg of serum albumin in 2 ml of isotonic sucrose. The phosphate bound to mitochondria in the absence of albumin is also shown for comparison (Table V, Experiment A'). For the reaction (Table V, Experiment B), 2.8 mg of mitochrome were substituted for the serum albumin. After 3 minutes at 0°, the mitochondria were centrifuged at $10,000 \times g$ and washed repeatedly with cold isotonic sucrose until the wash water approached background radioactivity levels, which required four washings. The mitochondria were then analyzed for radioactivity and protein nitrogen. It can be seen (Table V) that the addition of mitochrome to mitochondria \equiv P³² discharged almost 70 per cent of the bound phosphate to form inorganic phosphate.

Discharge of Mitochondrial Bound Nucleotide P³² by Mitochrome—Mito-

chondria with bound radioactive ATP (mitochondria-ATP³²) were prepared by mixing 4 ml of mitochondria (16 mg of protein N) in 0.25 M sucrose with 0.5 ml of serum albumin and 1 ml of phosphate-labeled

TABLE V
Discharge of Bound Inorganic and Nucleotide Phosphate from Mitochondria by Mitochrome

Experiment	Reaction time	Reaction mixture	Phosphate split from ATP	Phosphate bound to mitochondria	
				Inorganic	Nucleotide
	min		μmoles	μmoles per mg protein N	μmoles per mg protein N
A	3	Mitochondria \equiv P ³² + albumin at 0°		63.0	
A'	3	Mitochondria \equiv P ³² at 0°		52.0	
B	3	" " + mitochrome at 0°		20.0	
C	3	Mitochondria-ATP ³² at 0°		4.0	28.3
D	3	" " + mitochrome at 0°		1.2	13.6
E	2	Mitochondria-ATP ³² at 26°	1,600	56.9	21.1
F	2	" " + mitochrome at 26°	2,700	15.2	5.6
F'	7	" "	54,000	18.7	4.3
F''	12	" "	73,400	16.8	4.9

ATP³² (10 μmole = 1×10^5 c p m)³ The mitochondria were centrifuged at 0°, washed once with 0.25 M sucrose, and suspended in 4 ml of

³ ATP³² was prepared by the reaction of 0.023 M α -ketoglutarate, 0.0082 M MgCl₂, 0.016 M KCl, 0.0016 M NaHCO₃, 0.0029 M histidine buffer, pH 7.4, 0.0021 M AMP, 0.00146 M ATP, 4 mc of P³² in 0.0024 M inorganic phosphate, and 0.6 ml of mitochondria (2.5 per cent suspension in sucrose) in a total volume of 2.76 ml. After 30 minutes of aerobic shaking, the reaction in the Warburg flask was stopped with the addition of 1 ml of 20 per cent trichloroacetic acid and the protein precipitate was removed by centrifugation in the cold. The nucleotides were precipitated from the neutralized protein-free filtrate as the barium salt after addition of 10 mg of pure ATP as carrier. The barium nucleotides were dissolved in dilute acid, the barium was removed with Amberlite resin IR-100, and the effluent chromatographed over a Dowex 1 column according to the procedure of Cohn and Carter (25). The ATP fraction isolated was again concentrated by precipitation as the Ba salt. Analysis with myosin ATPase and with 7 minute hydrolysis in 1 N HCl showed that both labile phosphate groups were equally labeled with P³². Solution of the Ba ATP³² in dilute acid and removal of the Ba ion with IR-100 ion exchange resin gave stock preparations with less than 0.1 per cent of the total counts in the form of inorganic phosphate after being stored in the frozen state.

isotonic sucrose The control mixture contained 2.5 ml of 0.1 M Tris buffer, pH 7.4, 1 ml of mitochondria-ATP³² suspended in isotonic sucrose, and 6.5 ml of 0.25 M sucrose The reaction mixture had, in addition, 5.2 mg of mitochrome After equilibration at 0° for 3 minutes, the mitochondria were separated by centrifugation at 10,000 $\times g$, washed twice with 5 ml of 0.25 M sucrose, and then suspended in 5 ml of 3 per cent HClO₄ The distribution of P³² between inorganic and nucleotide phosphate in the deproteinized filtrate was determined by measuring the radioactivity of the isobutyl alcohol-benzene and water phases obtained with the Martin and Doty (26) procedure for phosphate analysis

At 0° comparatively little ATP is split by mitochondria even in the presence of mitochrome It is evident from Experiments C and D (Table V) that about 12 per cent of the total phosphate bound to mitochondria was analyzed as inorganic phosphate after acid denaturation of the mitochondria The rest remained as nucleotide phosphate Since the ATP³² used in the reaction contained less than 1 per cent inorganic P³², some of the ATP³² must have been converted to a bound phosphate ($\equiv P$) The addition of mitochrome to mitochondria-ATP³² discharged from the mitochondria about 70 per cent of $\equiv P$ and about 50 per cent of the phosphate bound as nucleotide

With the "static" equilibria relative to inorganic and nucleotide phosphate on the mitochondrial surface approximately defined, the nature of phosphate bound to mitochondria during the dynamic equilibria that result in dephosphorylation of ATP was of interest Experimental conditions for the dynamic equilibria are easily attained by merely raising the temperature of the reaction of mitochondria with excess ATP³² to 26° The reaction mixtures in Experiments E and F (Table V) contained 2.5 ml of 0.1 M Tris buffer, pH 7.4, 1 ml of mitochondria (4 mg of protein N) suspended in isotonic sucrose, 1 ml of ATP³² (12.4 μ moles), and 5.5 ml of isotonic sucrose The reaction mixture of Experiment F' had in addition 5.2 mg of mitochrome After the stipulated reaction times, the mitochondria were centrifuged and the total inorganic phosphate in the mixtures, as well as the phosphate bound on the mitochondria, were determined as described previously The data obtained are summarized in Experiments E, F, F', and F'' of Table V In the presence of excess ATP, with minimal net splitting to inorganic P, the nucleotide P and bound $\equiv P$ on mitochondria at 26° approximate the maximal values obtained at 0° in the static state With the addition of mitochrome, both inorganic P and nucleotide P are discharged from the mitochondria, accompanied by an increase in the total ATP hydrolyzed As the reaction proceeds with time, although there is a 50-fold increase in the inorganic P split from ATP, the amount of nucleotide P and $\equiv P$ bound to the mitochondria, as

well as the ratio of $\equiv P$ to nucleotide P, remains constant within the limits of the experimental error

Mitochrome $\equiv P$ —The discharge of a bound P from mitochondria by mitochrome and the activation of ATP dephosphorylation by mitochondria after the addition of mitochrome suggest the intermediate formation of a phosphorylated mitochrome as one possible explanation for the catalytic effect of mitochrome. Many proteins bind ions in a non-specific or "sta-

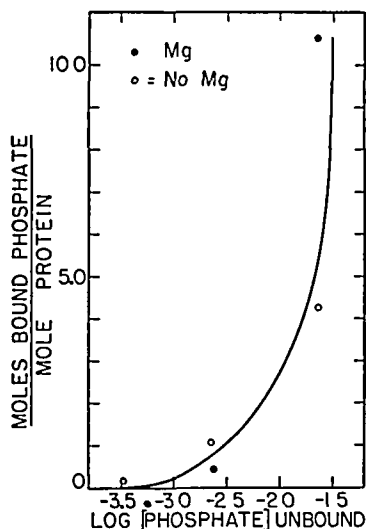


Fig 8 Binding of inorganic phosphate by mitochrome. 2 ml of solution containing 14.3 mg of mitochrome and 0.1 ml of 0.054 M PO_4 (1485 c.p.m. per ml = 1 μ mole of PO_4) were dialyzed against 50 ml of phosphate buffer, pH 7.3, at varying phosphate concentrations in the presence and absence of 0.001 M $MgCl_2$ for 48 hours with shaking at 5°. The contents inside and outside the dialysis bags were analyzed for inorganic phosphate and the radioactivity was determined on 1 ml aliquots by counting under conditions of constant geometry. The data are reported as the moles of phosphate bound per 100,000 gm of mitochrome plotted as ordinate against the log of the phosphate molarity outside the dialysis bag as abscissa.

tistical" manner that depends on the nature of the protein and varies primarily with the ionic strength of the environmental media. A more specific type of ion binding similar to enzyme-coenzyme compound formation takes place only at limited sites of the protein and changes the protein so uniquely that it takes on the characteristics of an active enzyme. The two types of protein and small molecule interaction are not necessarily exclusive of each other and may, in fact, act synergistically. With an anion-like phosphate, the possibility exists for a non-specific interaction of the ion with mitochrome as well as a specific interaction at a site or sites for the transfer of $\equiv P$. The data presented in Fig 8 demonstrate the

non-specific binding of phosphate by mitochromes both in the presence and absence of magnesium. Although this interaction would necessarily complicate any attempt to define by tracer techniques a mitochrome-phosphate compound formed in the dephosphorylation reaction, this difficulty is mitigated by the fact that the adsorbed phosphate is dissociated from the mitochrome at concentrations less than 0.001 M and can be removed from

TABLE VI

Phosphate Bound to Mitochrome during ATP Dephosphorylation with Mitochondria

Experiment No	Mitochrome added to reaction	Inorganic P formed	Mitochrome isolated	Bound P*	Atoms P / Mole mitochrome
	mg	γ	μ moles	μ atoms	
1	0.0	0.7	1.33	1.1	0.83
2	0.22	9.9	1.4	1.3	0.93
3	0.66	69.0	2.1	2.8	1.33
4	1.10	114.4	2.8	3.3	1.18
5†	1.13	32.0	8.8	8.3	0.94

Reactions in Experiments 1 to 4 were performed in 2.15 ml of 0.157 M sucrose and 0.0233 M Tris buffer, pH 7.4, containing 3.7 μ moles of ATP³² (50,000 c.p.m. per μ atom of P), 6.5 mg of mitochondria, and the mitochrome indicated. The reaction in Experiment 5 was performed in 4 ml of 0.213 M sucrose and 0.0325 M Tris containing 6.65 μ moles of ATP (177,200 c.p.m. per μ atom of P). After 10 minutes incubation at 26°, the mitochondria were removed by centrifugation (15,000 $\times g$) at 0° and the mitochrome in an aliquot of the supernatant fluid was precipitated by adjusting the solutions to contain 0.05 M MgCl₂ and 20 per cent alcohol at 0°. The precipitate was centrifuged and washed on a pad of hardened filter paper with 0.05 M MgCl₂ and 20 per cent alcohol until the counts in the filtrate reached background. The filter pad was air-dried and the radioactivity determined. Protein content of the sample was determined by micro-Kjeldahl analysis (13).

* Corrected for protein and counts found with the isolation procedure for mitochrome applied to zero time reactions of mitochondria in the absence of added mitochrome.

† The increased tonicity in this reaction mixture suppressed the diffusion of mitochrome from mitochondria but also inhibited the rate of dephosphorylation.

the protein. Isolation of mitochrome from the ATP-dephosphorylating reaction mixture after removing the mitochondria was accomplished by precipitating the protein at neutral or slightly acid pH in the presence of 0.05 M Mg and 20 per cent alcohol. When this precipitation is performed in the presence of inorganic P³², the mitochrome can be washed free of adsorbed phosphate.

The hypothesis of a mitochrome-phosphate intermediate became more plausible with the isolation from the ATP dephosphorylation reaction mixture with mitochrome of a protein with 1 atom of labeled phosphate per mole of mitochrome (Table VI). This composition was maintained

over a 5-fold concentration range of mitochrome with over a 100-fold increase in the splitting of P from ATP and under varying conditions of tonicity of the medium. Since the phosphate on the mitochrome could be recovered as inorganic phosphate after acid denaturation of the protein, and since the compound was not formed in the absence of mitochondria and ATP, it seems reasonable to assume that mitochrome \rightleftharpoons P was implicated in the dephosphorylation of ATP. This assumption must be tempered by cognizance of the possibility that mitochrome \rightleftharpoons P may represent a compound X-P that is released from mitochondria during the reaction and coprecipitates with the mitochrome. Until such a factor is demonstrated, the proposed hypothesis explains the data, albeit expediently.

DISCUSSION

Because of their obvious implications in a concept of cellular energy use, attention has been directed toward those reactions which produce an "uncoupling" of phosphorylation from oxidation in intact mitochondria. Our experiments show that mitochrome, a protein component of mitochondria, can act as an apparent "uncoupling" agent by activating a "latent ATPase," thereby inhibiting aerobic phosphorylation. The oxidative phosphorylation mechanism in mitochondria reacting with mitochrome remains essentially intact, since both recovery of phosphorylation and inhibition of ATPase activation are obtained when mitochrome is combined with serum albumin. The external action of mitochrome on mitochondria might be interpreted, then, as the introduction of an alternative pathway for the transfer of phosphate. Sufficient evidence is available from the tracer experiments to offer a framework for the appraisal of this pathway.

A simplified outline of the phosphate exchange equilibria on the surface of the mitochondrion is given in Fig. 9. Inorganic phosphate is rapidly taken up by mitochondria to form a bound phosphate (\rightleftharpoons P). The nature of this acid-labile phosphate bond is not known, but current views are that the phosphate is directly bound to protein (27). The point of interest is that mitochrome discharges this phosphate as inorganic phosphate presumably via a phosphate-mitochrome compound. When ATP³² is added to mitochondria, the P³² components bound to mitochondria are analyzed as ATP, ADP, and \rightleftharpoons P. Unless either \rightleftharpoons P or ADP is removed by some reaction, this apparent equilibrium might be considered as representative of a static state arising with mitochondria that show no ATPase activity. With the addition of mitochrome and the discharge of \rightleftharpoons P, the equilibrium would be displaced in the direction of inorganic P and a net dephosphorylation of ATP would occur. In this way the addition of mitochrome or the diffusion of mitochrome from mitochondria into the reaction media would, in effect, account for a "latent ATPase."

Albumin probably inhibits the decomposition of ATP by blocking the dephosphorylation of mitochondria $\rightleftharpoons P$ by mitochondria. Apparently the albumin-mitochondria interaction does not stop the transfer of $\rightleftharpoons P$ from mitochondria, since labeled mitochondria $\rightleftharpoons P$ can be separated from reaction mixtures containing albumin. However, the breakdown of mitochondria $\rightleftharpoons P$ to inorganic phosphate by mitochondria does not take place in the presence of albumin. A possible explanation is that albumin prevents the interaction of the mitochondria $\rightleftharpoons P$ with dephosphorylating sites on the mitochondria by steric hindrance or by a masking of functional groups on the protein. The activation of ATP dephosphorylation by mitochondria is also inhibited when the reactions are carried out in isotonic

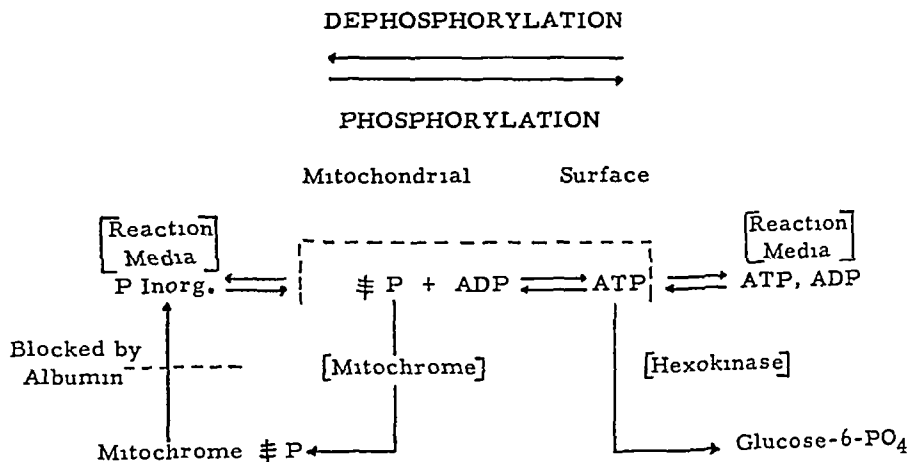


FIG 9 A scheme for the external action of mitochondria on the phosphate exchange equilibria of mitochondria. Symbols are defined in footnote 1.

sucrose, suggesting that the dephosphorylation sites on the mitochondria are less available under these conditions. It seems reasonable that these same sites are also made unavailable to the mitochondria by serum albumin when the reactions take place in the hypotonic solutions necessary to demonstrate maximal ATP splitting.

By establishing conditions for the removal of electrons from substrates and for the passage of these electrons along the electron transport chain of mitochondria, the reaction equilibria outlined in Fig 9 are shifted in the direction for the conversion of inorganic phosphate to ATP. This is enhanced when hexokinase is added to minimize the back-reaction by trapping phosphate as glucose 6-phosphate. It would appear, then, that hexokinase and external mitochondria act in opposition to each other. By discharging $\rightleftharpoons P$ and nucleotide from the surface of the mitochondria, mitochondria would make ATP less available for the hexokinase reaction and effect a decrease in oxidative phosphorylation efficiency. Conversely,

the same factors that inactivate ATP dephosphorylation when albumin is combined with mitochrome (*i.e.* shift equilibrium to the right) also operate to reestablish phosphorylation efficiency

Although the isolation of mitochrome made possible studies *in vitro* that helped explain certain properties of isolated mitochondria like the rapid loss of phosphorylation efficiency and the introduction of a "latent ATP-ase," it also opened the question of the physiological function of this new heme protein. Mitochrome exists primarily within mitochondria. It is difficult to conceive of its function there as an activator of ATP dephosphorylation, and it seems more reasonable to ascribe the external action of mitochrome to the fortuitous interaction with enzymes from which it is normally shielded. Instead, the presence of a heme group that can be reduced and reoxidized and its apparent involvement in a high energy phosphate transfer mechanism suggest that mitochrome may play an important role in the utilization of energy from the respiratory enzymes within mitochondria that is of greater interest and importance than its external action on mitochondria.

SUMMARY

A new chromoprotein, mitochrome, released from mitochondria after aging or other degradative processes, was isolated in purified form. From measurements of the partial specific volume, the diffusion constant, and the sedimentation constant, the molecular weight of mitochrome was estimated at 107,000 in agreement with the value of 102,000 calculated from the iron content of 0.055 per cent, assuming 1 atom of iron per mole of mitochrome.

Electrophoretic studies indicated a single component protein over the pH range both on the acid and alkaline side of the isoelectric point at pH 5.1.

Spectrophotometric analysis revealed two dominant bands for mitochrome at 280 and 410 m μ . The heme component was reduced by Na₂S₂O₄ with a concomitant shift of the absorption maximum to 422 m μ .

Electrophoretic and sedimentation experiments furnish evidence for a protein-protein interaction between mitochrome and serum albumin.

Equilibrium dialysis experiments indicate a binding of phosphate by mitochrome that depends on the concentration of the phosphate ion.

The addition of mitochrome to mitochondria reacting with substrate and hexokinase in a manner to demonstrate aerobic phosphorylation produced an inhibition of the amount of phosphate esterified that was proportional to the amount of mitochrome in the reaction. This inhibition was released by serum albumin.

The addition of mitochrome to mitochondria under anaerobic conditions with adenosine triphosphate as a substrate caused a net dephosphorylation.

of adenosine triphosphate to inorganic phosphate. This activation was inhibited by serum albumin.

Kinetic and radioactive tracer studies are interpreted to indicate that mitochrome functions as an activator for adenosine triphosphate dephosphorylation by discharging both phosphate and nucleotide from the surface of the mitochondria. The phosphate discharge takes place presumably through the intermediate formation of a phosphate-mitochrome compound. These reactions suggest that mitochrome acts externally on mitochondria by introducing an alternative pathway for the flow of high energy phosphate.

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THE METABOLISM OF HISTIDINE

I EFFECTS OF VITAMIN B₆ AND OF BIOTIN DEFICIENCY*

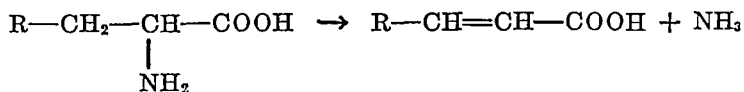
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Histidine is unique among the commonly occurring amino acids in that a major route (1) of its catabolism in the animal organism involves direct removal of the amino group to form an unsaturated compound, urocanic (imidazoleacrylic) acid, and ammonia. This may be considered as *straight* deamination in contrast to oxidative deamination and transamination reactions which have been described for most amino acids. In the latter reactions, riboflavin derivatives (2) and pyridoxal phosphate (3), respectively, have established coenzyme roles. Little is known, however, of the part that vitamin B derivatives play in straight deamination of amino acids.

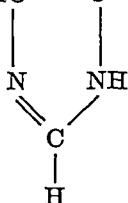
Biotin has been implicated as a cofactor of bacterial enzyme systems by which aspartic acid is deaminated to form fumaric acid (4). The action of aspartase in bacteria is analogous to that of histidase in mammalian and other species



where

R = HOOC— in aspartic acid (aspartase)

R = HC=C— in histidine (histidase)



Thayer and Horowitz (5) reported a several fold increase in the amount

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of L-amino acid oxidase produced by *Neurospora* grown in the presence of limited, as compared with higher, concentrations of biotin. The oxidase was more active toward histidine than toward other amino acids tested.

In view of these relationships of biotin and of the multiple role of vitamin B₆ derivatives in amino acid metabolism, it was of interest to investigate the effects of deficiencies of each vitamin on liver enzyme systems concerned with the straight deamination of histidine. In addition to histidase and urocanase, levels of aspartic-glutamic transaminase and rhodanese were measured. Rhodanese, which is not known to be linked to any major metabolic process, was assayed as an index of possible effects of the vitamin deficiencies on enzyme protein synthesis. The levels of transaminase provided an estimate of the degree of vitamin B₆ deficiency in the tissues of the animals fed diets lacking in this vitamin.

From these studies, evidence has been obtained that an adaptive increase in liver histidase accompanies depletion of vitamin B₆ or biotin in the tissues of the rat. It was also found that histidase, urocanase, and rhodanese activities per gm. of liver increase during normal growth.

EXPERIMENTAL

Pyridoxine Deficiency—Male, white rats of the Wistar strain (initial weight, 40 to 50 gm.) were fed a high protein (40 per cent casein) diet, devoid of pyridoxine, which contained deoxypyridoxine and isoniazide. The diet contained, in addition to the casein, in gm. per 100 gm., sucrose 45.8, powdered cellulose 5, salts (6) 2, corn oil 4, cod liver oil 2, choline 0.2, and vitamin mixture 1. In the vitamin mixture for the control animals were 50 mg. each of pyridoxine hydrochloride, thiamine hydrochloride, and riboflavin, 20 mg. each of biotin and folic acid, 200 mg. each of niacin, calcium pantothenate, and *p*-aminobenzoic acid, 20 gm. of inositol, and powdered sucrose to make 100 gm. The vitamin mixture used in the diet for the deficient animals was the same, except that pyridoxine was omitted and 100 mg. each of deoxypyridoxine and isoniazide were added per 100 gm. The intake per 10 gm. of food was thus 50 γ of pyridoxine hydrochloride or 100 γ of both of the antagonists. After 18 days of feeding of the deficiency diets, the usual symptoms (7) of pyridoxine deficiency were observed. The animals were killed at intervals during the following 10 days.

One group of animals was fed *ad libitum* up to a maximum of 10 gm. of food per day per rat. A second group was pair-fed. Several animals died in severe deficiency states. Data were obtained from ten control and five deficient animals fed *ad libitum* and from eight pairs of the pair-fed group.

Since the mean body weight of the deficient animals, at the time of

death, was less than half that of the control rats (54 gm *versus* 115 gm in the pair-feeding experiment), groups of normal rats of similar weight ratios were also studied. Twelve rats each were fed 10 gm per day of the control diet. Four of these animals were killed during 1 week to provide a group of average weight of 75 gm, the remainder were killed 2 or 3 weeks later, at which time the mean body weight was 164 gm.

Biotin Deficiency—Other male rats (initial weight 40 to 48 gm) were fed diets similar to that described for the pyridoxine-control animals, except that casein was replaced by commercial dried raw egg white. Biotin was omitted from the vitamin mixture used in preparation of the deficient diet for half of the rats. The remainder served as controls and were given 40 γ of biotin by intraperitoneal injection three times a week during the experimental period. The animals were pair-fed. After 7 weeks of dietary control, those which received no biotin exhibited various signs of biotin deficiency (8). Data were obtained from nine pairs. The average of the weights of the deficient animals at time of death during the 8th week was 122 gm, that of the control animals, 146 gm.

Assay Procedures—The rats were killed by decapitation. A portion of liver was taken for determination of total nitrogen (Kjeldahl method) and of dry weight (overnight at 105°). The remainder was ground with 5 volumes of cold 1 per cent KCl solution per gm of tissue in a Potter-Elvehjem homogenizer immersed in an ice bath. After removal of an aliquot for rhodanese determination, the homogenate was centrifuged at $6000 \times g$ for 10 minutes at 0°. Histidase, urocanase, and aspartic-glutamic transaminase were measured in the supernatant fluid.

The maximal rates of formation (at pH 9.2) and of disappearance (at pH 7.4) of urocanic acid, as measured at 277 m μ , the absorption maximum of the latter, were used to determine histidase and urocanase levels, respectively. Portions of supernatant material (0.05 or 0.10 ml) were incubated with histidine or urocanic acid and buffer in 1 cm Beckman cells as described by Mehler and Tabor (1).

For determination of transaminase activity, the coupled reaction scheme was utilized in which the rate of oxalacetate formation is followed by conversion of the latter to malate in the presence of excess malic dehydrogenase with the concomitant oxidation of reduced diphosphopyridine nucleotide (DPNH). The rate of disappearance of DPNH was measured at 340 m μ . The procedure and reagents were essentially those described by Karmen (9) for determination of serum transaminase, except that 2.0 ml of 0.1 M phosphate buffer, pH 7.4, were used in a total volume of 3.0 ml. 0.1 ml of a solution made by diluting the supernatant fluid 25-fold was used in place of serum.

For assay of histidase, urocanase, and aspartic-glutamic transaminase,

the temperature of incubation was 28°. Increase in temperature due to heat output from the hydrogen lamp of the Beckman DU spectrophotometer was minimized by insertion of the standard test tube attachment between the light source and cuvette holder. Preparations from deficient and corresponding control animals were usually analyzed simultaneously or within 1 hour of each other.

Rhodanese activities were measured by the colorimetric method of Cosby and Sumner adapted by Rosenthal *et al* (10), modified as suggested by Rosenthal and Vars (11) by the use of 0.1 M 2-amino-2-methyl-1,3-propanediol sulfate buffer, pH 8.8. 0.1 ml portions of one to five dilutions of the whole homogenates were used for analysis. Temperature of incubation for rhodanese was 20°.

Urocanic acid was made by enzymatic deamination of histidine with preparations from histidine-adapted strains of *Pseudomonas fluorescens* (1, 12). The molar extinction coefficients (at 277 m μ) of the products obtained from several runs were between 18,500 and 18,600. In subsequent calculations, the latter figure was utilized for histidase and urocanase, and the extinction coefficient for reduced diphosphopyridine nucleotide reported by Horecker and Kornberg (13) was used for transaminase activities.

Enzyme activities were calculated in terms of micromoles of substrate destroyed per minute per gm. of liver, a unit being the amount of enzyme which catalyzes the destruction of appropriate substrate at the rate of 1 μ mole per minute under the conditions used. Differences between the groups of animals were similar, whether activities were expressed as units per gm. of wet or dry weight or of total nitrogen of liver, or were based upon the protein content of the supernatant fluid from the homogenates. The averages of the dry weights of liver tissues from the pyridoxine-deficient rats and their pair-fed controls and from the biotin-deficient rats and the pair-fed controls were respectively 30.9, 29.3, 31.3, and 31.9 per cent, corresponding values for per cent total nitrogen were 3.67, 3.76, 3.85, and 3.90, averages for protein content of supernatant fluids, determined according to Lowry *et al* (14), were 18.2, 18.1, 19.3, and 18.4 mg. per ml., respectively.

Results

In Table I is a summary of the data obtained for histidase, urocanase, aspartic-glutamic transaminase, and rhodanese in the livers of vitamin B₆-deficient and control rats. Only histidase and urocanase were determined in the livers of the animals fed *ad libitum*. The results were so similar to those obtained with the pair-fed rats that they are presented together in Table I. Due to manipulative losses, values for aspartic-

glutamic transaminase and rhodanese were available from only seven of the eight pairs of rats of the pair-feeding experiment

The levels of histidase in the livers of the deficient animals are significantly greater than in livers of control rats. The depressed levels of transaminase are regarded as evidence of decreased effective amounts of tissue pyridoxal phosphate. The similarity of rhodanese and urocanase concentrations in both groups is interpreted to mean that liver enzyme protein synthesis was not generally affected by lack of pyridoxal phosphate.

Although the deficient animals were of the same age as the control rats,

TABLE I
Enzyme Levels in Livers of Vitamin B₆-Deficient and Control Rats

Enzyme	Diet	No of animals*	Range	Mean	P†
			<i>units per gm ‡</i>	<i>units per gm</i>	
Histidase	Control	18	0.232-0.426	0.315	<0.01
	Deficient	13	0.244-0.702	0.415	
Urocanase	Control	18	0.298-0.517	0.377	>0.05
	Deficient	13	0.236-0.458	0.362	
Aspartic-glutamic transaminase	Control	7	36.1-77.0	51.6	<0.01
	Deficient	7	10.6-21.4	18.1	
Rhodanese	Control	7	496-731	605	>0.05
	Deficient	7	480-623	547	

* These numbers include both rats which were fed *ad libitum* and those which were pair-fed for histidase and urocanase, and pair-fed rats for transaminase and rhodanese.

† Probability level according to Fisher's "t" test (15)

‡ Micromoles of substrate destroyed per minute per gm of liver (see the text) histidase, pH 9.2, 28°, urocanase, pH 7.4, 28°, transaminase, pH 7.4, 28°, rhodanese, pH 8.8, 20°

they weighed considerably less. The elevated levels of liver histidase, however, cannot be ascribed to differences in body weight. As shown in Table II, concentrations of this enzyme, as well as of urocanase and rhodanese, are normally lower in smaller than in larger (older) rats. Aspartic-glutamic transaminase activities per gm of tissue did not change significantly with age during the period studied.

In Table III are comparable data for biotin-deficient and pair-fed control rats. Histidase levels were also somewhat elevated in the livers of animals deficient in biotin. The differences are significant at a probability level of between 1 and 5 per cent, according to Fisher's "t" test (15). Urocanase, aspartic-glutamic transaminase, and rhodanese activities were not significantly different from control values.

TABLE II
Enzyme Content of Rat Liver

Weight of rat	Histidase	Urocanase	Aspartic glutamic transaminase	Rhodanese	Total nitrogen
<i>gm</i>	<i>unit per gm *</i>	<i>unit per gm</i>	<i>units per gm</i>	<i>units per gm</i>	<i>per cent</i>
64	0 188	0 184	53 4	363	2 71
71	0 230	0 294	46 8	483	3 49
80	0 221	0 267	61 2	517	3 53
87	0 184	0 267	58 4	361	3 13
Mean	0 206	0 253	55 0	431	3 22
142	0 291	0 348	66 7	575	3 66
148	0 392	0 405	47 9	799	3 84
148	0 396	0 524	58 0	834	3 98
164	0 315	0 353	42 8	651	3 48
174	0 304	0 396	51 6	549	3 44
176	0 322	0 396	44 9	578	3 40
180	0 386	0 469	55 7	750	3 95
183	0 386	0 405	49 9	674	3 67
Mean	0 349	0 412	52 2	676	3 68

* Micromoles of substrate destroyed per minute per gm of liver (see the text) histidase, pH 9.2, 28°, urocanase, pH 7.4, 28°, transaminase, pH 7.4, 28°, rhodanese, pH 8.8, 20°

TABLE III
*Enzyme Levels in Livers of Biotin-Deficient and Pair-Fed Control Rats**

Enzyme	Diet	Range	Mean	P†
		<i>units per gm ‡</i>	<i>units per gm</i>	
Histidase	Control	0 171-0 325	0 241	<0.05, >0.01
	Deficient	0 208-0 462	0 303	
Urocanase	Control	0 213-0 342	0 284	>0.05
	Deficient	0 207-0 421	0 314	
Aspartic-glutamic transaminase	Control	26.5-80.9	50.7	>0.05
	Deficient	40.5-74.2	57.1	
Rhodanese	Control	557-750	669	>0.05
	Deficient	468-744	645	

* Nine pairs

† Probability level according to Fisher's "t" test (15)

‡ Micromoles of substrate destroyed per minute per gm of liver (see the text) histidase, pH 9.2, 28°, urocanase, pH 7.4, 28°, transaminase, pH 7.4, 28°, rhodanese, pH 8.2, 20°

The disparity between mean enzyme concentrations in the livers of the two sets of control animals of the vitamin B₆ and biotin deficiency studies is believed to be related to the dietary protein sources used. This question will be considered in a subsequent paper.

DISCUSSION

Dietary vitamin B₆ is required for maintenance of the liver enzyme system by which histidine undergoes transamination in the rat (16). The observed elevation in histidase levels in the livers of the pyridoxine-deficient animals is interpreted as an adaptive increase in this enzyme which catalyzes straight deamination in response to denial of a route of degradation of histidine which is pyridoxal phosphate-dependent, *i e*, transamination.

The increase in histidase levels in the livers of rats made deficient in biotin is analogous to the adaptive increase in L-amino acid oxidase production by *Neurospora* grown in media which contained limited amounts of this vitamin. These findings would seem to implicate biotin in some, as yet undiscovered, role in amino acid metabolism.

In early work, Edlbacher and Becker (17) observed increased levels of histidase in livers of thiamine-deficient animals. Conflicting reports (18, 19) have appeared from Japanese workers as to the effect of folic acid in activation of partially purified preparations of liver histidase. Ichihara *et al* (20) have reported a summary of experiments in which liver histidase was decreased in rats fed diets containing Aminopterin or which were low in folic acid. Addition of folic acid and glutathione to crude enzyme preparations from these animals restored activity to levels found in control rats. The data were based upon disappearance of histidine in various incubation mixtures rather than on the rate of formation of urocanic acid.

It is of interest that histidase, urocanase, and rhodanese are increased to a greater extent than the total liver nitrogen during rapid growth, whereas aspartic-glutamic transaminase levels remain unchanged. The observed increase in histidase is in agreement with the findings of Ross and Ely (21). Cohen and Hekhuis (22) found higher levels of transaminase in the livers of older, as compared with younger, cats. Beaton *et al* (23) reported that liver aspartic-glutamic transaminase levels increased with age, however, the rats in their study were older (*i e*, weighed more initially) than the ones used in the present work.

SUMMARY

1. Significantly elevated levels of histidase accompanied decreased levels of aspartic-glutamic (and presumably of histidine) transaminase in the livers of vitamin B₆-deficient rats. Urocanase and rhodanese concentrations were little different from control values.

2 Liver histidase levels were also higher in biotin-deficient than in corresponding control rats (with a difference significant at a probability level of between 5 and 1 per cent) No significant difference was noted in aspartic-glutamic acid transaminase, in urocanase, or in rhodanese concentrations

3 Concentrations of liver histidase, urocanase, and rhodanese, but not of aspartic-glutamic transaminase, increase during rapid growth of the rat

4 Changes in enzyme activity are discussed in terms of metabolic adaptation to depletion of tissue cofactors

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SERUM LIPIDE ANALYSIS BY CHROMATOGRAPHY AND INFRARED SPECTROPHOTOMETRY*

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In an earlier paper (1) we discussed the infrared absorption spectra of some lipoproteins and related lipides, and described a crude analytical procedure which included infrared spectrophotometric measurements of the main lipoprotein constituents which were separable by simple solvent extraction. To place this method on a firmer, more useful basis, one of the steps proposed was to achieve a better separation of the lipide components by the use of chromatography. We have attempted this with use of silicic acid-Celite adsorption columns in essentially the manner described by Borgstrom (2). Analysis of the eluted fractions by infrared absorption measurements provides a useful complement to the chromatographic separation, and the combination of these two techniques provides an integrated system of lipide analysis. Brief outlines of the method have been given elsewhere (3, 4), and the results obtained in the study of lipoprotein composition have also been presented (5).

The method is capable of yielding much information from a single relatively small lipide sample by the use of a set of uniform operations. For appropriate purposes, and with due consideration of the requirements of accuracy, it can replace several separate chemical determinations, *ie*, total fatty acids, free fatty acids, total cholesterol, free cholesterol, and lipide phosphorus. In so far as degradation can be avoided during handling, the method is non-destructive of the lipide components, which are still available for other investigations or tests. We have applied the procedure with some success in studying the compositions of samples as small as 5 mg of total lipide extracted from serum or from serum fractions and further reduction in scale appears to be possible.

The accuracy obtainable is subject to some variation among the various components, depending on the amount present and, to some extent, on the composition of the mixture. Specific factors bearing on the accuracy will be pointed out later, but it may be indicated here that ± 10 per cent is a rough working estimate for the probable error for any component present.

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to the extent of at least 0.5 mg and which comprises more than 10 per cent of the total lipide

If a measurement of only one lipide component (*e g* cholesterol) is desired, the present method offers no advantage over established chemical methods. Glycerides may be an important exception, however, since they are measured directly as esters in a fraction from which other classes of esters have been separated.

The estimation of total phosphatides in serum has been reasonably consistent with lipide phosphorus determinations. Work is in progress on a detailed analysis of this class of lipides, and information should be obtained relative to the validity of this measurement for more variable types of phosphatide distributions. Until such information becomes available, the application of this method to other lipide systems, such as tissue extracts, should be made with caution.

EXPERIMENTAL

The procedure will be described for the treatment of a 1 ml serum sample, ordinarily yielding 5 to 10 mg of total lipides. For larger samples the operations should be scaled up proportionately with respect to size of column, quantity of adsorbent, and volumes of extracting and eluting solvents. (A 2-fold variation in amount of lipide for a given set of column conditions is apparently tolerable with respect to the quoted over-all accuracy of the method.)

Materials—All solvents used were of good reagent grade. Ethyl ether was freed from peroxide. The Celite was analytical Filter-Aid, Johns-Manville. The silicic acid was c.p. precipitated.

Purity of solvents can be checked by recording the infrared spectra of their non-volatile residues. Complete solvent blanks may also be run and the absorbances determined at the analytical wave lengths for each eluted fraction. Corrections may be applied if necessary, or further purification carried out if indicated.

The adsorbent is prepared by sifting and mixing together thoroughly 2 parts (by weight) of silicic acid and 1 part of Celite, washing the mixture with methanol, drying it, and finally heating it to 120° for 24 hours. It may be stored in a closed vessel or, preferably, in a desiccator.

Extraction of Lipides—Any method which extracts the lipides completely would presumably be satisfactory, except that, in order to attain complete extraction, considerable amounts of non-lipide substances are also extracted. Excessive amounts of such impurities are undesirable, and some provision should be made for removing them. The two-phase method described below appears to be satisfactory in removing these impurities, and it also allows acidification to insure that free fatty acids are extracted in the

carboxyl form (If the latter consideration is of no interest, acidification may be omitted)

The procedure is as follows 4 ml of methanol are measured into a clean 40 ml screw-capped vial, the cap being lined with a thin disk of polyethylene or Teflon 1 ml of serum is pipetted slowly into the methanol, which is gently shaken and swirled The vial is placed in a hot water bath or heating block at 50–60° for about 15 minutes After cooling, 5 ml of water and 20 ml of ethyl ether are added The vial is then tightly capped and shaken in a mechanical shaker for 4 minutes The phases are separated by centrifugation and the ether layer is siphoned off into a 125 ml Erlenmeyer flask The aqueous layer is acidified with 6 drops of 6 N HCl, and the extraction is twice repeated with 15 ml of ethyl ether The combined ether extracts are evaporated to dryness at room temperature The maintenance of a nitrogen atmosphere during this and subsequent evaporations does not appear to be necessary for the present level of accuracy but is a desirable precautionary measure, especially if tests other than infrared measurements are contemplated (see "Results and discussion")

Chromatography—For a 5 to 10 mg lipide sample, the type of adsorption column used consists of a vertical glass tube about 15 cm long and 5 mm in inner diameter, with the lower end drawn to a fine tip and plugged with glass wool The adsorbent (0.25 to 0.30 gm) is added most conveniently as a thin suspension in hexane The amount can be controlled with sufficient accuracy by determining the required column height (about 35 mm) and adjusting it to that level Packing is accomplished by the application of pressure from a rubber bulb or from a low pressure nitrogen or air line During this step, and throughout the remainder of the chromatographic operation, the solvent level should not be allowed to fall below the top of the adsorbent bed The sample is transferred quantitatively to the column with three or four small portions of hexane The final rinsing of the flask may be made with the first elution solvent, 5 per cent chloroform in hexane 8 ml of 5 per cent chloroform in hexane are then passed through the column to elute Fraction I, which is collected in a 15 ml centrifuge tube (For this sized column we have found it convenient to couple the top of the tube to a 10 ml syringe containing the required volume of solvent The coupling consists of a rubber serum bottle closure which fits the top of the tubing as a cap This is pierced with a short hypodermic needle and mounted in a plastic frame in the proper orientation, so that the column fits on the under side with the needle projecting a short distance down into it The syringe is fitted to the needle from above and is held in vertical alignment by a ring or clip A small brass weight, recessed on the bottom to fit over the plunger of the syringe, serves to apply pressure to the column Since the system is closed,

the solvent in the column stops flowing after the plunger reaches the bottom of the cylinder. Accidental drying out of the adsorbent is thereby effectively prevented, and close attention to the solvent level is not required.)

After the elution of Fraction I, Fraction II is eluted with 8 ml of CHCl_3 . Fraction III is eluted into a third tube with 8 ml of methanol. All the fractions are evaporated to dryness under a hood at a temperature not exceeding 50° . A current of nitrogen may be used to hasten the evaporation. Fraction III is often contaminated with a small amount of adsorbent particles from the column which may become evident before or during evaporation. This is removed by centrifugation after about half of the methanol has evaporated. After evaporation, it is necessary to rinse down the walls of the tubes carefully with small amounts of chloroform and again to evaporate to dryness. If it is not possible to proceed with the infrared measurements within a few hours, the samples should be stored in a vacuum desiccator.

Infrared Measurements—All of our measurements have been made with a Baird Associates double beam recording spectrophotometer equipped with a sodium chloride prism. The absorption cell used has an optical path of 0.9 mm and a volume of 0.15 ml.

For infrared measurement, the sample (eluted fraction) is brought into solution in a known volume of carbon disulfide,¹ and transferred to the absorption cell. The measurement of solution volume is made in one of two ways, depending upon the sample size. The larger samples, amounting to about 2 mg or more, are quantitatively transferred to calibrated volumetric tubes of 0.5, 1.0, or 2.0 ml capacity by using capillary pipettes and small volumes of carbon disulfide. With the smaller samples (1 mg or less), a graduated 0.2 ml pipette can be employed as the measuring device. Approximately 0.2 ml of carbon disulfide is added to the sample in the centrifuge tube, and the tube is swirled and agitated. The solution is drawn up completely into the pipette, the volume is noted, and the cell is filled directly from the pipette. It is helpful to have the tip of the pipette drawn or ground to a size to fit the cell opening. The pipette is operated by a 0.25 ml syringe to which it is connected by plastic tubing.

The spectrum of each eluted fraction, in a measured volume of CS_2 , is recorded from 5 to $11\ \mu$. Over the bands whose absorbances are to be measured, the scanning rate should be slow enough to allow full response of the recorder. In our instrument, speeds of 2 to 3 minutes per micron have been used. The use of a cell containing pure solvent in the reference beam is optional, since, for the desired precision, it is necessary in either case to run background curves of solvent contained in the sample cell.

¹ All operations with CS_2 should be carried out under a hood.

Absorbances at the specified absorption maxima are calculated in the customary way as $\log_{10} (T_0/T_s)$, where T_0 = transmittance of the solvent and T_s = transmittance of the sample solution

Typical spectra obtained for the chromatographic eluates of a serum lipide sample are shown in Fig 1 (The region from about 6 to 7.5 μ is

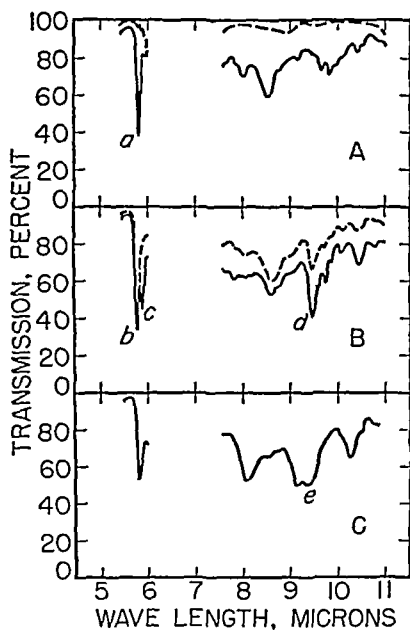


Fig 1 Infrared spectra of serum lipid fractions eluted from silicic acid-Celite, cell thickness, 0.9 mm. A, solid line, Fraction I, cholesteryl esters, 5.2 mg in 1.0 ml of CS_2 , broken line, CS_2 transmission curve. B, solid line, Fraction II, glycerides, 0.87 mg, free fatty acids, 0.36 mg, cholesterol, 1.53 mg, volume of CS_2 , 0.2 ml, broken line is a similar fraction in which free fatty acids are not present in sufficient amounts to give a discrete band at 5.85 μ . C, Fraction III, phosphatides, 2.28 mg in 0.5 ml of CS_2 . The lower case letters indicate the principal bands used for measurement.

obscured by a strong absorption band of CS_2 and has been deleted) The dotted curve in Fig 1, A is the solvent transmission curve

Fraction I (Fig 1, A) consists of cholesteryl esters. Their concentration is determined by measuring the peak absorbance of either the 5.8 μ band or the 8.55 μ band and reading from the appropriate calibration curve (absorbance *versus* concentration, see below)

Fraction II (Fig 1, B) contains glycerides, unesterified fatty acids, and unesterified cholesterol. For three components it is necessary, in general, to use three absorption bands, each calibrated for all three components. In this instance the problem is simplified in that the absorption of cholesterol at 5.75 and 5.85 μ is extremely small and can be neglected. These

two absorption bands can therefore be used to calculate fat (glycerides) and fatty acids as a two-component system. This is most conveniently done by means of linear simultaneous equations (see Mellon (6)), provided the Beer's law relationship holds. Briefly, the calculation involves first writing the equations for the absorbances A_1 and A_2 (at wave lengths 1 and 2) as functions of the concentrations and the slopes ($a = A/C$) of the calibration curves

$$A_1 = a_{11}C_1 + a_{12}C_2$$

$$A_2 = a_{21}C_1 + a_{22}C_2$$

The cell thickness is a constant term implicit in all of the a values

Solution of these equations gives explicit expressions for the concentration in terms of the measured absorbances and a set of constants derived from the a values

$$C_1 = k_1A_1 + k_2A_2$$

$$C_2 = k_3A_1 + k_4A_2$$

If we substitute a typical set of calibration values, the equations become

$$A_{5.75\mu} = 0.100 C_F + 0.016 C_{FA}$$

$$A_{5.85\mu} = 0.009 C_F + 0.166 C_{FA}$$

where C_F = concentration of fat and C_{FA} = concentration of fatty acids. The solutions in terms of numerical constants and measured absorbances are

$$C_F = 10.07 A_{5.75} - 0.97 A_{5.85}$$

$$C_{FA} = -0.55 A_{5.75} + 6.09 A_{5.85}$$

Such a set of equations need be obtained only once for a given set of calibration data and may be used as long as the slopes of the A versus C curves do not change.

After determining the concentrations of fat and fatty acids, the contributions of these two components to the absorbance at 9.5μ are calculated from their calibration curves at that wave length. These quantities are applied as corrections to the measured absorbance of the cholesterol peak. In the particular cell for which the above calibration data were obtained, the A/C values at 9.5μ were 0.010 and 0.006 for fat and fatty acids, respectively. Thus, for example,

$$A_{9.5}(\text{corrected}) = A_{9.5}(\text{measured}) - 0.01 C_F - 0.006 C_{FA}$$

By using this corrected value, the concentration of cholesterol can be found from its calibration curve. The low order of accuracy in the determination

of this component results partly from the fact that the measured absorbance at $9.5\ \mu$ is usually small (for the conditions described), and partly from the necessity for applying a double correction.

If the amount of free fatty acids is so small that no absorption peak at $5.85\ \mu$ is resolved from the glyceride band, the measurement of absorbance at that point becomes inaccurate. However, it is possible to make an estimate of the upper limit of free fatty acids and ignore their contribution to the glyceride band at $5.75\ \mu$. The absorbance of that band can be used directly for the estimation of glycerides from the appropriate calibration curve. Only one correction need then be applied to the $9.5\ \mu$ cholesterol band.

Fraction III contains the phosphatides, and their total concentration is determined from the absorbance of the $9.35\ \mu$ band.

Once the concentration of any component in the cell has been found from the infrared measurements, that value is multiplied by the volume of CS_2 used. This gives the actual weight of that component, *i.e.*, the weight of the component = the concentration in cell \times volume of CS_2 . The concentration of the component in the original sample, *e.g.* in mg per 100 ml, is given by $\text{mg per cent} = (\text{mg} \times 100)/(\text{volume of sample})$.

Calibration—The spectra of the substances used as reference standards are shown in Figs 2 and 3, A. The principal calibration bands are indicated by lower case letters, which serve to identify the bands with the corresponding ones in the lipid fraction spectra in Fig 1 and with the calibration curves in Fig 4.

In order to prepare calibration curves (absorbance *versus* concentration), it is necessary to choose some representative lipide of each class as a standard and to demonstrate that reasonable or anticipated variations in structure or fatty acid composition do not strain the limits of accuracy imposed by other methodological factors. Of the components being measured, only cholesterol can be dealt with as a specific chemical compound.

With fatty acids, for example, one uses the $5.85\ \mu$ absorption band, which is a measure of carboxyl groups. Hence it is necessary, just as in titration, either to express the results on a molar basis or to make assumptions about the nature of the mixed fatty acids and their average molecular weight. We have chosen the latter alternative in using oleic acid as a calibration substance since its molecular weight is reasonably close to an average for the fatty acids found in extracted serum lipides. We have verified that calibration curves for lauric, palmitic, stearic, and elaidic acids are all in substantial agreement with the oleic acid curve when their slopes are adjusted by using molecular weight ratios as multiplying factors.

Similarly, the 5.75 and 5.8 μ carbonyl absorption bands are measures of ester groups. We have used commercial olive oil as a representative triglyceride and find satisfactory conformity with its calibration curve by

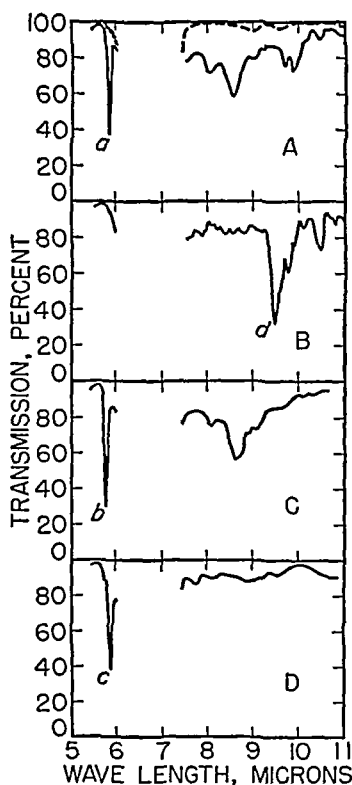


FIG 2

FIG 2 Infrared spectra of non-phosphatide reference materials from 5 to 11 μ CS_2 solutions, cell thickness, 0.9 mm. The lower case letters indicate the principal bands used for measurement. A, cholesteryl laurate, 7.16 mg per ml, m.p. 77-78°, prepared by esterification of cholesterol with lauric acid. B, cholesterol, m.p. 149°, repurified via dibromide. 11.57 mg per ml. C, olive oil (commercial), 4.96 mg per ml. D, oleic acid (Hormel Institute), 2.50 mg per ml. The broken line in Fig. A is a CS_2 transmission curve.

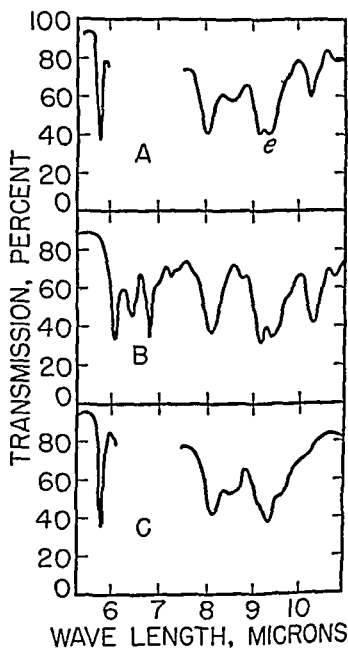


FIG 3

FIG 3 Infrared spectra of phosphatide constituents from 5 to 11 μ , cell thickness, 0.9 mm for A and C. A, egg phosphatides, reprecipitated several times with acetone, 4.17 per cent P, 6.26 mg per ml in CS_2 . The band designated e corresponds to the calibration curve in Fig. 4. B, sphingomyelin (H. E. Carter), solid film between salt plates. C, cephalin, prepared from egg phosphatides by the chromatographic procedure of Lea, Rhodes, and Stoll (11), 4.0 per cent P, 5.06 mg per ml in CS_2 .

tripalmitin, tristearin, corn oil, coconut oil, and tung oil, again making the necessary adjustments for molecular weight. Since our standard for cholesteryl esters has been synthetic cholesteryl laurate, the slope of the curve obtained for this substance has been adjusted to correspond to

cholesteryl oleate Substantially the same curve is obtained in a similar way from either cholesteryl palmitate or cholesteryl stearate

Calibration for total phosphatides is contingent upon the existence of an infrared absorption band that occurs with approximately the same intensity in the spectra of the principal component phosphatides From a qualitative examination of the spectra in Fig 3, it is evident that regions of absorption at $8.1\ \mu$ and 9.1 to $9.4\ \mu$ are potentially suitable for this purpose Both of these regions are believed to be associated with phosphoric ester groups (7) (It should be noted that the $5.8\ \mu$ ester carbonyl band is not found in the sphingomyelin spectrum, and the $10.3\ \mu$ band is not present in the cephalin curve) Since lecithin is the major component, we have used either of its two absorption maxima at 9.15 and $9.35\ \mu$ as the analytical wave length (The $9.15\ \mu$ band was used in certain cases in which it was desirable to use CHCl_3 as a solvent) Until recently egg lecithin, prepared from egg yolks and purified by repeated precipitation with acetone, has been used as a calibration standard A typical preparation of this material contained 4.17 per cent P and about 80 per cent phosphatidyl choline recoverable by the alumina column treatment of Hanahan *et al* (8) Recently it has been possible to calibrate with synthetic dioleylecithin² and to compare its spectrum with that of egg phosphatides Their absorptivities differed by 10 per cent at $9.15\ \mu$ and by only 3 per cent at $9.35\ \mu$ These differences are consistent with the presence of cephalin in the egg material, since, as may be seen in Fig 3, C, the cephalin spectrum in this region exhibits a single peak at $9.3\ \mu$ rather than the doublet pattern of the lecithin curve The measured absorptivity of this isolated cephalin fraction at $9.35\ \mu$ (slightly off the peak) is the same as that of lecithin Because purified sphingomyelin² has a limited solubility in CS_2 , it can be compared only in chloroform solution at $9.15\ \mu$ From limited data, its absorptivity at that wave length is about 20 per cent less than that of lecithin Qualitative comparison of the curves of solid films in this region suggests that the intensity correspondence should be about the same at both wave lengths Use of the lecithin absorptivity to measure total phosphatides leads to a 4 per cent error if the sphingomyelin content is assumed to be approximately 20 per cent With present calibration data and over-all measurement accuracy, it is not considered worth while to use a weighted absorptivity for the mixed phosphatides From the empirical standpoint, the validity of the infrared measurement is upheld by comparison with lipide phosphorus determinations in serum (Table III)

A typical set of calibration curves is illustrated in Fig 4 Varying

² We are extremely grateful to Professor Erich Baer of the University of Toronto for the synthetic dioleylecithin, and to Professor H. E. Carter of the University of Illinois for the sphingomyelin

degrees of non-linearity are apparent in them, which are of no consequence when the graphs are read directly. For absorbance values up to about 0.5, the deviations from linearity are small, and straight line approximations up to that value have been used to obtain the slopes required for the two-component calculation described above. Very high absorbances, as well as very low ones, are to be avoided as less accurate on general spectrophotometric principles. This may sometimes necessitate repetition of the infrared measurement at a difference concentration in cases in which the estimate of required CS_2 volume fails to give a concentration for which the absorbance falls in the range of about 0.1 to 0.7.

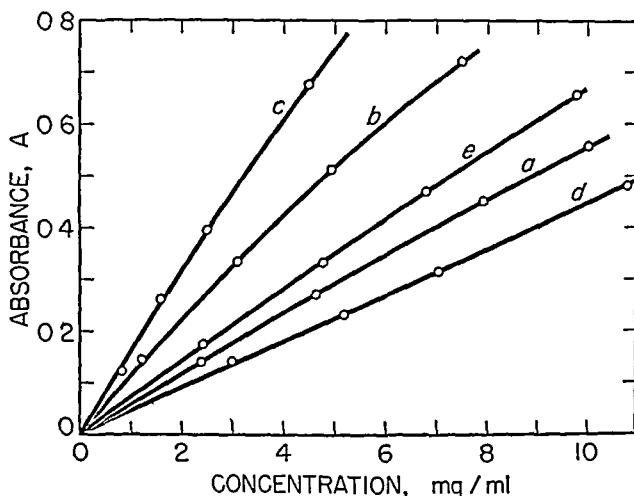


FIG. 4 Absorbance *versus* concentration curves at the principal wave lengths for the reference materials in CS_2 solution. *a* = cholesteryl laurate, $5.8\ \mu$ (corrected to cholesteryl oleate, see the text), *b* = olive oil, $5.75\ \mu$, *c* = oleic acid, $5.85\ \mu$, *d* = cholesterol, $9.5\ \mu$, *e* = egg phosphatides, $9.35\ \mu$.

The calibration values should be checked periodically. We have found them to remain stable within a few per cent over periods of several months, although they are subject to variation from changes in cell characteristics or instrumental conditions if these are not well controlled.

RESULTS AND DISCUSSION

The effectiveness of the chromatographic separation was verified in two ways. First the procedure was carried out with a known mixture, simulating serum lipides, and with a lipide extract from human serum lipoprotein (*S*₆ specifies a lipoprotein that has a flotation rate of 6 Svedberg units in a salt solution of density 1.063) both on 1 gm columns. The samples were about 25 mg each. Fig. 5 shows an elution curve obtained by evaporating successive 2 ml fractions to dryness, redissolving each in 0.2 ml of

hexane, and measuring their refractive indices. The curve is a plot of Δn ($= n_{\text{solution}} - n_{\text{solvent}}$) versus the cumulative volume of each elution solvent, i.e., ordinate points are plotted at 2 ml intervals of abscissa X , and correspond to the measurement for the 2 ml volume fraction from $X-2$ to X (n designates the refractive index).

A second verification of the separability under these conditions is given in Table I. These results were obtained from three analyses of triplicate 12.3 mg aliquots of a total lipid extract from serum. In this instance 1 gm columns were used, and some of the conditions for elution were altered to show the effects of these variations on the over-all results. For

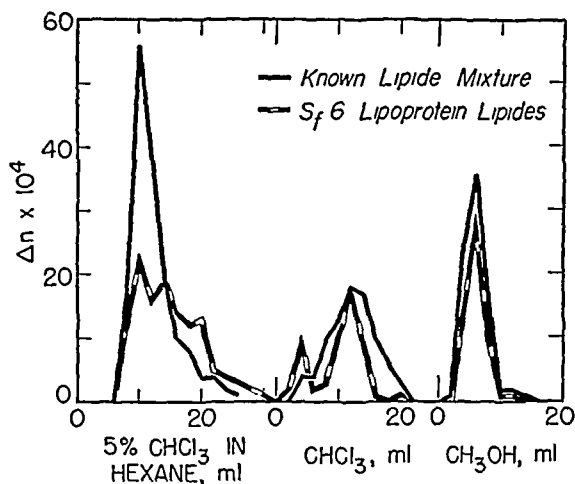


Fig 5 Lipide elution curves obtained by refractive index measurements. 25 mg samples, 1 gm columns. 2 ml fractions collected, evaporated to dryness, redissolved in 0.2 ml of hexane for refractive index measurement. $\Delta n = n_{\text{solution}} - n_{\text{solvent}}$

all columns the elution of Fraction I was broken into several subfractions in order to obtain a rough distribution curve. Furthermore, this elution was carried well beyond the standard elution volume (32 ml for a 1 gm column). Fraction II was broken into only two subfractions, with a 25 per cent extension of the standard volume. No breakdown was made of Fraction III. Weight recovery and total phosphatide values check well in all three analyses. From the reproducibility of the individual components it seems evident that the net results are but little affected by the variations employed. The chromatographic operating conditions chosen as standards are therefore considered to be adequate and to have some latitude, at least for the level of accuracy attained at present.

In certain applications a procedure essentially like that just described has been employed as a more precise control of the separation of Fractions

degrees of non-linearity are apparent in them, which are of no consequence when the graphs are read directly. For absorbance values up to about 0.5, the deviations from linearity are small, and straight line approximations up to that value have been used to obtain the slopes required for the two-component calculation described above. Very high absorbances, as well as very low ones, are to be avoided as less accurate on general spectrophotometric principles. This may sometimes necessitate repetition of the infrared measurement at a difference concentration in cases in which the estimate of required CS_2 volume fails to give a concentration for which the absorbance falls in the range of about 0.1 to 0.7.

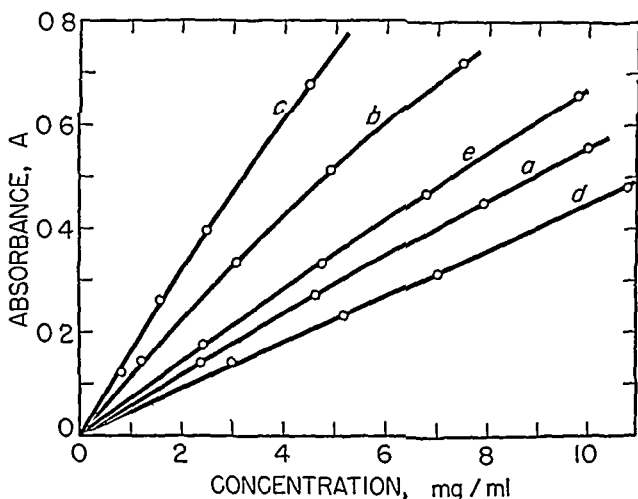


FIG. 4 Absorbance *versus* concentration curves at the principal wave lengths for the reference materials in CS_2 solution: *a* = cholesteryl laurate, $5.8\ \mu$ (corrected to cholesteryl oleate, see the text), *b* = olive oil, $5.75\ \mu$, *c* = oleic acid, $5.85\ \mu$, *d* = cholesterol, $9.5\ \mu$, *e* = egg phosphatides, $9.35\ \mu$.

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Table III gives the results obtained in the complete analyses of some randomly selected serum samples, run in a routine manner as three different sets at different times. Total cholesterol and total phosphatide values obtained by this method are compared with chemical determinations of the same components. The samples had been analyzed for total cholesterol as a routine measurement in connection with other studies in progress in this laboratory. The modified Schoenheimer-Sperry method used has been described by Colman and McPhee (9). From chromatographic-infrared measurements, total cholesterol is calculated as the sum of unesterified cholesterol plus $0.60 \times$ cholesteryl esters. Phosphorus was determined in lipid extracts from 0.1 ml serum aliquots by an adaptation of the

TABLE II

Quintuplicate Analyses of Known Mixture of Lipides

Values are in mg

Component	Amount present	Amount found					Average error
		Analy-sis 1	Analy-sis 2	Analy-sis 3	Analy-sis 4	Analy-sis 5	
Cholesteryl esters	2.09	1.83	1.95	1.92	2.05	1.99	per cent 6.8
Unesterified cholesterol	0.32	0.26	0.32	0.28	0.23	0.28	14.1
Glycerides	0.53	0.55	0.60	0.52	0.53	0.53	3.8
Unesterified fatty acids	0.21	0.20	0.21	0.21	0.21	0.20	2
Phosphatides (egg)	1.14	1.23	1.33	1.22	1.21	1.18	8.2
Average error for all components							7.0

method of Griswold, Humoller, and McIntyre (10). The factor 25 was used to convert phosphorus to equivalent phosphatide. In two sets of duplicates (Samples 10a, 10b, 11a, and 11b) the agreement for all components is well within 10 per cent.

Sources of Error—Consideration of the most likely sources of error may not only suggest possible improvements but may also help to avoid pitfalls. The principal errors can be segregated as they arise in extraction, chromatography, infrared photometric measurement, or calibration.

The extraction procedure described has been demonstrated to yield 96 per cent or more of the total lipides of serum, *i.e.*, upon saponification of the extracted residue, an amount of fatty acid can be recovered that is 4 per cent or less of the weighed extract. From most lipoproteins, from which the bulk of serum proteins have been separated, the unextracted lipid is usually less than 1 per cent. Other extraction methods may prove to be equally good or superior.

As indicated by the data in Table I and Fig 5, the chromatographic separation is generally satisfactory. Small amounts of cross contamination between Fractions I and II are difficult to detect in the spectra, since the

TABLE III

Results of Chromatographic Infrared Serum Lipide Analyses Comparison of Phosphatides and Total Cholesterol with Chemically Determined Values

All concentrations are in mg per 100 ml of serum

Sample No	CE	UC	GL	UFA	PH	Chemical PH	Δ PH, per cent	Total cholesterol		
								Calculated in infrared absorption	Chemical	Δ , per cent
1	220	32	79	35	154	180	-14	163	141	+16
2	321	54	194	33	243	238	+2	245	239	+3
3	402	70	155	27	258	261	-1	309	333	-7
4	236	26	145	23	181	192	-6	167	173	-4
5	246	40	48	25	174	195	-11	188	209	-10
6	202	41	84	29	174	174	0	162	179	-10
7	272	63	147	9	254	256	-1	226	243	-7
8	510	91	124	23	327	350	-7	397	428	-7
9	101	24	93	21	140	139	+1	85	111	-23
10a	317	52	82	21	209	206	+2	241	241	0
10b	300	50	78	21	209	184	+13	229	241	-5
11a	397	67	104	50	269	249	+8	303	321	-6
11b	392	63	100	47	273	252	+8	296	321	-8
12	200	29	37	46	163	152	+7	148	162	-9
13	267	42	100	31	196	195	+1	201	231	-13
14	257	36	45	38	181	196	-8	189	191	-1
15	259	40	110	33	209	196	+7	194	216	-10

CE = cholesteryl esters, UC = unesterified cholesterol, GL = glycerides (presumably triglycerides), UFA = unesterified fatty acids, and PH = phosphatides. Analyses were made at different times in three sets, comprised of Samples 1 to 4, 5 to 9, and 10 to 15. In the set comprised of Samples 5 to 9, the sums of lipides measured by infrared absorption were compared with the weighed total lipide extracts. The calculated values ranged from 94 to 97 per cent of the weighed amounts. Samples 10a, 10b, 11a, and 11b are pairs of duplicates.

principal ester group band positions of cholesteryl esters are very close to the corresponding ones of glycerides. The differences are sufficient, nevertheless, that gross overlapping of these components causes measurable shifts from the correct band positions. Premature elution of Fraction II constituents in Fraction I has not been observed in the total serum lipide samples so far encountered. It has occurred, but infrequently, for other lipide mixtures containing high relative proportions of glycerides, appar-

ently by exceeding the capacity of the column. There is, for occasional samples, evidence of cholesteryl esters being carried over into Fraction II. The amount involved is ordinarily a small percentage of the total cholesteryl esters and is only detectable if the amount of glycerides is small. Therefore, it may in such cases represent a larger relative error in glycerides if that component is calculated from the total ester absorption. A carry-over of cholesteryl esters could conceivably occur as a result of oxidation, since inert atmospheres have not customarily been used in our work. Upon again chromatographing cholesteryl ester fractions that have been exposed to air in the dry state for long periods of time (weeks), it is found that they are distributed over all fractions. Whether rigorous exclusion of oxygen from the samples during evaporation and handling would improve the apparent over-all accuracy of the method has not been investigated.

No measurable phosphorus has been found in Fraction II, but on the other hand Fraction III may contain as much as 10 per cent of non-phosphatide material. This material has not been well characterized as yet, but it contains a heme-like pigment and has a considerably lower absorptivity than the major phosphatides at 9.35μ . The estimated error in total phosphatides due to its presence is about 3 per cent or less.

The concentration error resulting from infrared measurement error has been estimated as about 4 per cent. The precision of the photometric measurements would presumably be improved if they were made at stationary wave length settings instead of from recorded curves. However, during the developmental stage it has been helpful to have an extended spectral region in which to verify qualitatively the identities of the separated components and to recognize accidental contaminations. (Fraction II has occasionally been found to contain phthalate esters, probably originating from impure solvents or accidentally extracted from poorly protected vial cap liners.)

In the determination of calibration curves, the error can be made small by averaging several measurements at each concentration. A greater inaccuracy undoubtedly arises from the arbitrary choice of standards, as discussed under calibration. This is a necessary compromise involving a degree of non-validity that is difficult to assess without more detailed information concerning the compositions of the individual lipid fractions.

SUMMARY

A tentative method has been developed for the analysis of serum lipides by using chromatography and infrared spectrophotometry. The extracted lipides are separated into three fractions by successive elutions from a silicic acid-Celite column with chloroform-hexane (1:19), chloroform, and

methanol By suitable infrared absorption measurements of these fractions (redissolved in carbon disulfide), the amounts of cholesteryl esters, glycerides, total phosphatides, cholesterol, and free fatty acids can be estimated The accuracy for a given component is somewhat dependent on the composition of the sample In general, the probable error is about ± 10 per cent for the major components, and slightly greater for unesterified cholesterol and fatty acids

We wish to thank Dr Hardin B Jones and Dr John W Gofman for their continued interest and support Lipide phosphorus analyses were performed by Gary Nelson

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ON THE ACETYLATION OF ALIPHATIC AMINES BY KIDNEY PREPARATIONS*

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The feeding of *p*-aminobenzoic or α -amino- γ -phenylbutyric acid to rats results in the excretion of acetylaminobenzoic or acetylphenylaminobutyric acid in the urine. Simultaneous feeding of isotopically labeled compounds such as acetic or pyruvic acid (1, 2), D- or L-alanine (3), D- or L-serine (4), or an acetylamino¹ acid (5) gives rise to labeled acetyl groups. Whereas most combinations of acetyl precursors and amines give nearly identical results, the isotope concentration of acetylphenylaminobutyric acid is much higher if labeled D-alanine, D-serine, or acetylglycine is fed. To explain these findings, it was suggested (3) that acetylation of phenylaminobutyric acid occurs in part in the kidney (6), and it was further assumed that D-alanine and acetylglycine are converted to acetate in the kidney with little dilution. In contrast, other acetyl precursors are predominantly metabolized in the liver where the metabolic pool of acetate is large. (1) *p*-Aminobenzoic acid (7) is acetylated only in the liver.

The *in vitro* studies reported here support this hypothesis and indicate that acetylation in the kidney may be due to a reversal of hydrolysis catalyzed by the enzyme acylase (8), in contrast to a transfer reaction which appears to be the mechanism of acetylation in the liver (9).

Results

The acetyl acceptor amine used in these experiments was cyclohexyl-L-alanine. Its optically active form is prepared conveniently by catalytic hydrogenation of L-tyrosine. This compound is acetylated *in vivo* (Table I) in the same manner as the α -amino- γ -phenylbutyric acid previously¹ used.

In Table II are given the quantities of cyclohexylalanine and *p*-aminobenzoic acid acetylated by the various labeled compounds in kidney and liver slices. In agreement with the experiments *in vivo*, it was found that cyclohexylalanine was readily acetylated in kidney slices if either acetate, D-alanine, or acetylglycine was the source of label, whereas L-alanine yielded a smaller amount of acetylamine. *p*-Aminobenzoic acid is not

* This work was supported by a grant from the United States Public Health Service.

¹ Labeled in the acetyl moiety only.

acetylated in kidney slices Cyclohexylalanine is acetylated by kidney slices to a somewhat greater extent than by liver slices In liver slices,

TABLE I
Acetylation of Foreign Amines in Vivo

Labeled precursor fed	Acetyl groups	
	Acetyl- α amino- γ phenylbutyric acid	Acetyl cyclohexyl L-alanine
	<i>RIC/Q</i>	<i>RIC/Q</i>
	<i>per cent</i>	<i>per cent</i>
1-C ¹⁴ -Acetic acid	4.3*†	2.8†
2-C ¹⁴ -Pyruvic acid	2.5*†	1.6†
2-C ¹⁴ -L-Alanine	1.9†	1.8†
2-C ¹⁴ -D-Alanine	10†	14†
1-C ¹⁴ -Acetylglycine	35†-67§	58

RIC = relative isotope concentration

* Averaged values

† *Q* = 0.4 to 1.0 mmole per 100 gm per day

‡ *Q* = 0.4 mmole per 100 gm per day, amine, 0.4 mmole per 100 gm per day

§ *Q* = 0.4 mmole per 100 gm per day, amine, 0.1 mmole per 100 gm per day

|| *Q* = 0.45 mmole per 100 gm per day, amine, 0.2 mmole per 100 gm per day

TABLE II
Acetylaminines Formed by Slices in Vitro

Tissue and amine	Amount of acetylamine formed per gm slice with			
	0.32 mmole 1-C ¹⁴ acetic acid	0.42 mmole 2-C ¹⁴ L alanine	0.49 mmole 2-C ¹⁴ D alanine	0.5 mmole 1-C ¹⁴ acetylglycine*
	μ moles	μ mole	μ moles	μ moles
Kidney + CHA	3.5†	0.62	2.5	3.5
" + pABA	0.1	0.03		
Liver + CHA	0.94	0.47	0.64	0.44
" + pABA	0.66	0.32		0.30

* Isotope in acetyl moiety

† In a separate experiment, 4.1 and 5.4 μ moles were formed with 0.32 and 0.44 mmole of 1-C¹⁴-acetic acid

1.5 gm of liver slices or 0.75 gm of kidney slices was incubated for 4 hours at 37° in 24 ml of Krebs-Ringer bicarbonate buffer, containing 0.24 mmole of amine, 56 μ moles of unlabeled acetylamine were added as carrier after incubation CHA = cyclohexylalanine, pABA = *p*-aminobenzoic acid

cyclohexylalanine and *p*-aminobenzoic acid were acetylated to approximately the same extent, acetate being the most efficient source of the acetyl groups

The activity of kidney minces in Ringer-phosphate buffer was found to be only slightly less than that of the slices. The quantity of acetylcyclohexylalanine formed in minces increases approximately linearly with time for the first 4 hours of incubation. The yield of acetylamine depends upon the cyclohexylalanine concentration below 0.003 M but is nearly independent of the amine concentration above this value. The amounts produced, depending on the concentration of acetate or acetyl glycine, are given in Fig. 1. Heating of the mince to 100° for 5 minutes abolished the reaction, the addition of NaF (0.04 M), KCN (0.04 M), NaN_3 (0.04 M), or 2,4-dinitrophenol (0.003 M) does not change the yield of acetylcyclohexyl-

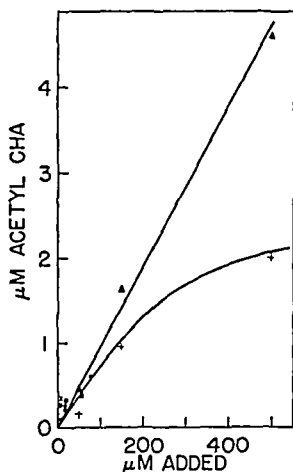


Fig. 1 Quantity of acetyl cyclohexylalanine formed by rat kidney minces in five separate experiments. ▲, ●, and ■ = acetate, + and × = acetyl glycine

alanine. Incubation under nitrogen reduced acetylation in some instances, but not consistently.

Only one-tenth to one-fifth of the original activity was recovered from rat or calf kidneys disintegrated in a Waring blender or ground with powdered glass (Experiment 2, Table III). Water extracts of rat kidney acetone powders are as active as the homogenates (Experiment 3, Table III). The enzyme can be concentrated and partially purified by ammonium sulfate fractionation as follows. Calf kidney acetone powder was extracted with 2 volumes of water and centrifuged at $4000 \times g$ for 30 minutes at 0°. To the supernatant fluid ammonium sulfate was added and the fraction precipitated between 30 and 50 per cent saturation was collected. The precipitate was dissolved in 0.1 volume of water, dialyzed for 4 hours against water at 0°, and centrifuged at $10,000 \times g$ for 1 hour at 0°. Precipitation with ammonium sulfate and dialysis and high speed centrifugation were repeated. For every gm of acetone powder, 27 mg of final product were obtained. This preparation is stable for at least 48 hours.

at room temperature, is inactivated by heating to 100° for 5 minutes, and requires no additions other than the substrates and buffer (Experiment 4,

TABLE III
Formation of Acetylcyclohexyl-L-alanine by Kidney Preparations

Experiment No		Experimental conditions	Acetylamine formed μmoles
1a	0.7 gm rat kidney	Slices	5.8
1b	0.7 " " "	Mince	4.9
2a	0.7 " " "	Slices	3.3
2b	0.7 " " "	Homogenate (Waring blender)	0.24
3a	0.1 " " "	Acetone powder, water extract dialyzed and centrifuged	0.17
3b	0.1 " calf "	" "	0.22
4a	As in Experiment 3b		0.25
4b	" " " 3b	30% (NH ₄) ₂ SO ₄ fraction	0.04
4c	" " " 3b	30-50% " "	0.48
4d	" " " 3b	Supernatant	0.01
5a	As in Experiment 4c	100 μmoles C ¹⁴ -potassium acetate	0.10
5b	" " " 4c	100 μmoles C ¹⁴ -acetylglycine	0.08
5c	" " " 4c	100 " C ¹⁴ -potassium acetate + 100 μmoles acetylglycine	0.13
5d	" " " 4c	100 μmoles C ¹⁴ -acetylglycine + 100 μmoles potassium acetate	0.05
6a	2.7 mg prepared as in Experiment 4c, heated 5 min to 100°		0.01-0.05*
6b	2.7 mg prepared as in Experiment 4c		0.30-0.60*
6c	1 mg acylase		0.17
6d	10 mg acylase		0.75

*Range for ten individual experiments Experiment 1 Krebs-Ringer phosphate buffer, 240 μmoles of CHA, 440 μmoles of C¹⁴-KAC, total volume, 24 ml, O₂, 4 hours, 37°, 56 μmoles of carrier Experiment 2 as in Experiment 1, but 330 μmoles of C¹⁴-KAC Experiment 3 100 μmoles of C¹⁴-KAC, 100 μmoles of CHA, 20 μmoles of collidine buffer (pH = 6.8), total volume, 4 ml, air, 3 hours, 37°, 100 μmoles of carrier Experiment 4 as in Experiment 3, but 15 μmoles of phosphate buffer (pH = 7.4), total volume, 9 ml Experiment 5 as in Experiment 4, but 20 μmoles of phosphate buffer (pH = 7.4) Experiments 6c and 6d as in Experiment 5, but total volume, 3.1 ml

Table III) Acetylation is maximal at pH 7 to 7.5, negligible below pH 6 and above pH 9, and independent of the nature of the buffer. The following compounds alone or in combination have no significant effect on

the quantity of acetylamine formed Mg^{++} (0.002 M), ATP (0.0006 to 0.005 M), CoA (0.03 to 3 units per ml), NaF (0.001 M), CH_3COOH (0.001 M), KCN (0.01 M), NaN_3 (0.07 M), and 2,4-dinitrophenol (0.003 M). If labeled acetylglutamate is used, the quantity of acetylcyclohexylalanine formed is the same as when labeled acetate is added. Unlabeled acetylglutamate does not decrease the formation of labeled acetyl groups from acetate but unlabeled acetate decreases the acetylation from labeled acetylglutamate (Experiment 5, Table III). A partially purified acylase² preparation shows approximately the same acetylating power per gm of material as the 30 to 50 per cent $(NH_4)_2SO_4$ fraction prepared from calf kidney (Experiment 6, Table III). Hydrolysis of acetylcyclohexylalanine by acylase has been demonstrated previously (10).

DISCUSSION

Acetylation by kidney preparations differs from liver (3) in several respects: the specificity in regard to the kind of amine, the change of activity on disruption of the cellular structure, and the conditions for enzyme activity.

Kidney slices appear to be able to acetylate only aliphatic amines, whereas in the liver aromatic amines too are acetylated, as can be seen from Table II, in agreement with previous observations *in vivo* (6).

The amount of acetylcyclohexylalanine formed in kidney minces is as much as 100 times the amount found in cell-free extracts. Either the enzyme catalyzing acetylation in kidney is so labile that it is destroyed upon disintegration of the cells or intact kidney cells can actively excrete the acetylamine acid, thereby displacing the equilibrium in the synthetic direction. In view of the insensitivity of the acetylation reaction in kidney minces to anaerobic conditions or to respiratory poisons, the source of energy for such an excretion process is at present undetermined.

An enzyme system present in cell-free kidney extracts catalyzes the reaction between amino acids and acetate. It has been partially purified and its activity is not affected by dialysis, the addition of metabolic inhibitors, or a number of known coenzymes. This acetylating enzyme is found in the same protein fraction as acylase (11), and acylase preparations possess a similar acetylating activity. For these reasons the acetylating enzyme in cell-free kidney extracts appears to be identical with acylase. However, an additional acetylating enzyme in intact kidney cells different from acylase cannot be excluded.

Acetylation with direct transfer of acetyl groups was not observed (5). In minces acetate is more effective than acetylglutamate. In cell-free prep-

² The authors are indebted to Mr. L. L. Lachat and Mr. M. A. Mitz of Armour and Company, Chicago, for this material as well as for various kidney preparations.

ations acetylation by labeled acetylglycine is depressed by unlabeled acetate whereas that by labeled acetate is unaffected by normal acetylglycine

EXPERIMENTAL

Syntheses—The preparation of acetic acid-1- C^{14} has been reported (12) Cyclohexyl-L-alanine was prepared by hydrogenation of L-tyrosine with the Adams catalyst and hydrogen (13) The crude product was recrystallized as the hydrochloride from 10 per cent hydrochloric acid $[\alpha]_D = 12.9^\circ$ in 4 per cent hydrochloric acid Acetylcyclohexyl-L-alanine was obtained by treating the amino acid dissolved in sodium hydroxide with acetic anhydride and recrystallizing it from water, m p 202° , $[\alpha]_D = 3.1^\circ$ in ethanol and -5.2° in 0.86 N sodium hydroxide

Incubation Procedures—Liver and kidney slices were cut freehand Kidney minces were prepared by forcing the kidneys through a 20 mesh stainless steel screen Slices and minces were incubated in a 125 ml Erlenmeyer flask with shaking at 37° in Krebs-Ringer phosphate buffer (14) Homogenates, prepared in a Waring blender, and acetone powder extracts were incubated in test tubes at 37° Phosphate buffer was used in most experiments

The amount of acetylcyclohexylalanine produced was estimated by the isotope dilution method (15) After incubation in the presence of carboxyl-labeled acetate, unlabeled acetylcyclohexylalanine was added, the mixture acidified immediately with sulfuric acid³ to pH 2, extracted with ether, and the ether solution reextracted with 1 M $KHCO_3$ The latter solution was then acidified and extracted with ether, and the ether solution was washed twice with water containing 10 per cent acetic and 1 per cent sulfuric acids, in order to dilute any labeled acetic acid present in the ether extract The residue from the ether solution was recrystallized from water with charcoal until the melting point reached $200-201^\circ$ and the C^{14} concentration remained constant The quantity of acetylamine formed, Q , was calculated by the following formula $Q = a/(b - a) \times C$, where C = the quantity of carrier added, a = the isotope concentration of the isolated acetyl group, and b = the isotope concentration of the added acetic acid

SUMMARY

1 Cyclohexyl-L-alanine is acetylated *in vivo* in the same manner as γ -phenyl L- α -aminobutyric acid

2 Rat liver slices acetylate both cyclohexyl-L-alanine and *p*-amino-

³ If the incubation is carried out at a pH of 5 or below, no C^{14} is found in the isolated acetylcyclohexylalanine This result excludes the exchange of the C^{14} -acetic acid with the acetyl group of the carrier

benzoic acid, whereas rat kidney slices acetylate cyclohexyl-L-alanine only, in agreement with previous observations *in vivo*

3 A soluble enzyme obtained from rat or calf kidney catalyzes the formation of acetylcyclohexyl-L-alanine from acetate. This reaction appears to be a reversal of hydrolysis, and the enzyme seems identical with acylase. The implications of this finding are discussed.

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MECHANISM OF BORATE INHIBITION OF DIPHENOL OXIDATION BY TYROSINASE*

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(Received for publication, August 20, 1956)

Borate inhibits alkaline phosphatase (1) and xanthine oxidase (2) competitively by reacting with the enzymes. Although borates form complexes with catechol (3), Winfield (4) and Klein (5) have been unable to demonstrate inhibition of diphenol oxidation by tyrosinase in borate solutions. Positive evidence has been obtained in the present work which demonstrates that borate does inhibit diphenol oxidation. Corroborative evidence was obtained from the finding that the borate-diphenol complex has an absorption peak in the ultraviolet region and that there is a direct correlation between the complex and the observed inhibition.

EXPERIMENTAL

Mushroom tyrosinase of high purity was purchased from the Treemond and Reheis Corporation, the preparations containing 2000 and 1700 Miller-Dawson catecholase units per mg. of dry weight, respectively.

The enzymatic activity was determined in the presence and the absence of ascorbic acid. When ascorbic acid was used, the rate of oxidation of the substrate was determined by the rate of ascorbic acid oxidation at 265 m μ . The ascorbic acid concentration was 0.1 mM, whereas the diphenol concentration was varied from 0.002 mM to 0.36 mM. When dihydroxyphenylalanine was used as a substrate in the absence of ascorbic acid, the enzymatic activity was determined by the formation of dopachrome at 305 m μ . The stock tyrosinase solution was diluted with distilled water before use, and about 3 catecholase units were used.

Spectrophotometric measurements were made with a Beckman DU spectrophotometer with 1 cm. quartz cuvettes, and the experiments were carried out at room temperature.

* Prepared from a thesis submitted by Kerry Yasunobu for the degree of Doctor of Philosophy, June, 1954.

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‡ Deceased March 30, 1952.

Results

Effect of Variation in Substrate and Borate Concentrations

Determinations of enzyme activity were made on various substrates with and without borate. When the reciprocal of the reaction velocity is

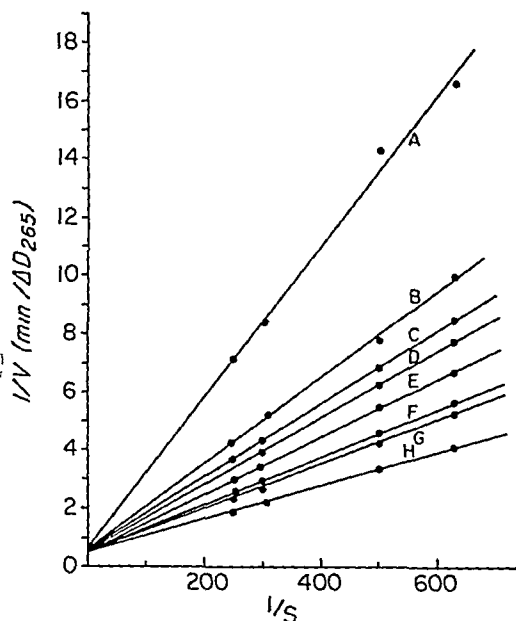


FIG 1

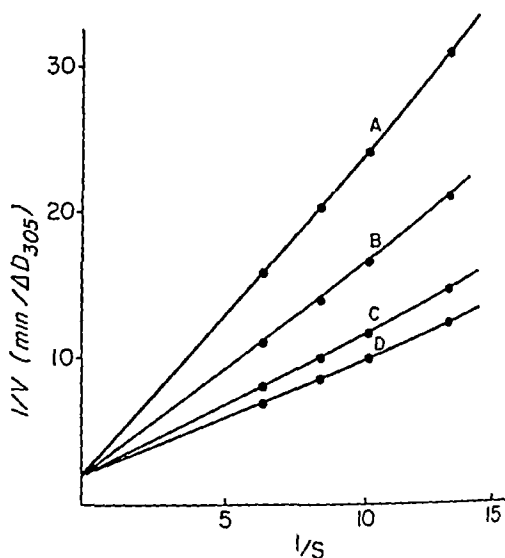


FIG 2

FIG 1 Effect of variation in catechol, borate concentration, and pH. In Curve A, experiments were carried out at pH 8.0 and with 6.7 mM borate, in Curve B, at pH 8.0 and with 3.3 mM borate, in Curve C at pH 7.0 and in 133 mM borate, in Curve D, at pH 6.0 and with 333 mM borate, in Curve E, at pH 8.0 and with 133 mM borate, in Curve F, at pH 6.0 and with 133 mM borate, in Curve G, at pH 7.0 and with 6.7 mM borate, in Curve H at pH 6, 7, or 8 with zero borate.

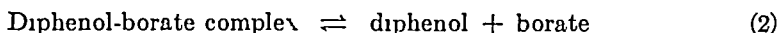
FIG 2 Effect of variation in dopa and borate concentration and pH. In Curve A, experiments were conducted at pH 7.8 and with 3.3 mM borate, in Curve B, at pH 7.0 and with 9.99 mM borate, in Curve C, at pH 6.0 and with 66.6 mM borate, and in Curve D, at pH 6, 7, or 7.8 and with zero borate.

plotted against the reciprocal of the substrate concentration, the curves shown in Figs 1 and 2 are obtained. For the reaction system, ascorbic acid, diphenol, and enzyme, the activity was measured as the rate of change in optical density at 265 mμ per minute. Fig 1 shows the results for catechol. Miltzer (6) reports that borate does not react with ascorbic acid, but, to demonstrate that the inhibition by borate is not due to formation of a complex with ascorbic acid, the activity was measured at 305 mμ without ascorbic acid for dihydroxyphenylalanine and enzyme. In Fig 2 are given the results for dihydroxyphenylalanine and tyrosinase and

the inhibition is clearly demonstrated. If it is assumed that a rapid reversible equilibrium exists between the inhibitor, substrate, and substrate-inhibitor complex, and if the concentration of the last complex is small compared to that of the first, then Equation 1 may be readily obtained

$$\frac{1}{v} = \left(1 + \frac{I}{K_i}\right) \frac{K_m}{V_m S} + \frac{1}{V_m} \quad (1)$$

It will be shown later that the inhibitor combines with the substrate so that K_i , in contrast to the usual case (7), is the equilibrium constant for the following reaction



Effect of pH—The ability of borate to inhibit tyrosinase activity depends markedly upon the pH, as shown in Figs 1 to 3. A much higher concentration of borate is required at pH 5.7 than in more alkaline solutions. Tyrosinase activity in the absence of inhibitor is not appreciably affected by pH over the range studied. Boric acid is a very weak acid and, if the assumption is made that it is only the H_2BO_3^- ion which forms the complex with the diphenol, the results are consistent. Concentrations of the H_2BO_3^- were calculated by using 6.4×10^{-10} as the ionization constant for the first hydrogen of boric acid. In Fig 4 is seen the correlation between the inhibition and the calculated concentration of the H_2BO_3^- ion. In these curves, the pH effect shown in Fig 3 for total borate has disappeared.

Evidence for Diphenol-Borate Complex—Boeskin (3) demonstrated the existence of catechol-borate complexes from conductivity studies. In our work, by use of buffered solutions, we discovered that the addition of boric acid to catechol and dihydroxyphenylalanine solutions resulted in increased absorption and in shifts to slightly higher wave lengths. Moreover, in agreement with Boeskin, who found no increase in conductivity for resorcinol-borate or hydroquinone-borate solutions, there was no increased absorption or spectral shift when borate was added to these compounds. Addition of borate to ferulic acid also resulted in no change in absorption. It appears from these examples that borate forms complexes only with unsubstituted orthodiphenols. The equilibrium constant for the formation of the complex, K

$$\begin{aligned} \text{Diphenol} + \text{H}_2\text{BO}_3^- &\rightleftharpoons \text{complex} \\ K &= \frac{(\text{complex})}{(\text{diphenol})(\text{H}_2\text{BO}_3^-)} \end{aligned} \quad (3)$$

was calculated from the increase in absorption with increasing concentrations of borate at constant diphenol concentration. Fig 5 illustrates the

increase in absorption at $286\text{ m}\mu$ for dopa, with increasing borate concentrations at pH 7.8. Fig. 6 shows the spectral changes for 0.1 mM catechol at pH 5.7 and 7.8 and that higher concentrations of borate are required in acid solutions.

The value for the equilibrium constant was obtained by plotting $\Delta D_{286\text{ m}\mu}/$

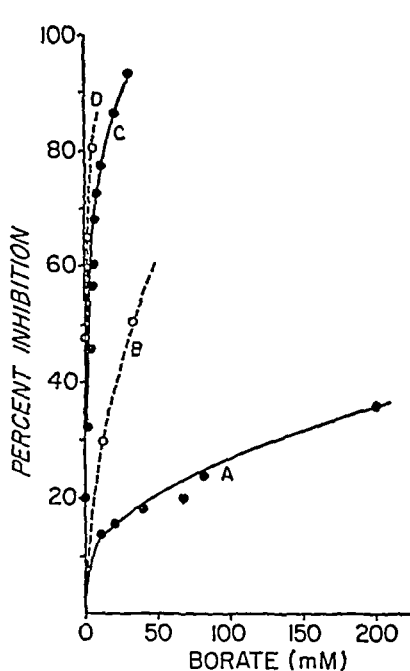


FIG. 3

FIG. 3 Variation in per cent inhibition with varying boric acid concentration. ●, experiments with dopa (0.1 mM), ○, catechol (0.0032 mM with 0.1 mM ascorbic acid). In Curves A and B, experiments were carried out at pH 5.7, in Curves C and D, at pH 7.8.

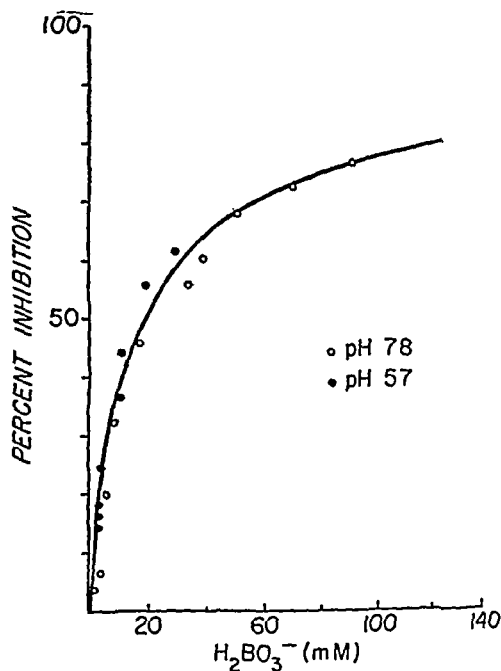


FIG. 4

FIG. 4 Variation in per cent inhibition with varying H_2BO_3^- concentration. Experiments with dihydroxyphenylalanine. ●, experiments at pH 5.7, ○, pH 7.8. Dihydroxyphenylalanine concentration was kept constant at 0.1 mM.

(H_3BO_3) as the ordinate and $\Delta D_{286\text{ m}\mu}$ as the abscissa, the value of the slope of the resulting line giving K directly. The equilibrium constant was also calculated by using the molecular extinction coefficient for the complex, which is about 4860 at $286\text{ m}\mu$, and by substituting the appropriate values in the case of dihydroxyphenylalanine. The K values are listed in Table I.

Correlation between Spectral and Enzymatic Data

When the per cent inhibition and $\Delta D_{286\text{ m}\mu}$ were plotted as the ordinate and the H_3BO_3 concentration as the abscissa, the two curves obtained were

almost superimposable, and it was evident that there was a direct correlation between the spectral increase in absorption and the inhibition. To test the relationship more closely, by use of the constant obtained spectrophotometrically for dihydroxyphenylalanine and borate while the total diphenol and borate concentrations were varied, calculations were made so

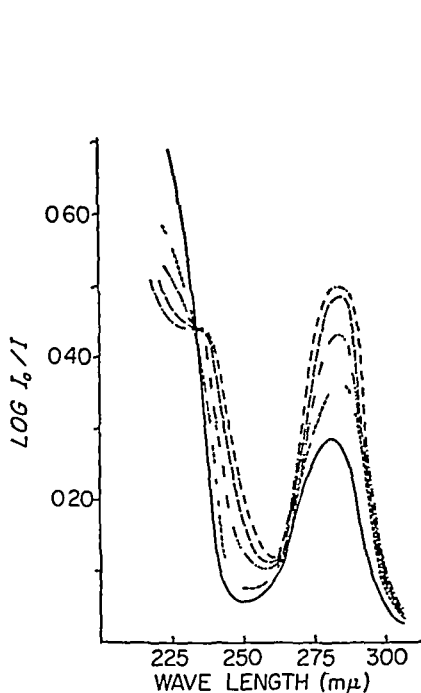


FIG 5

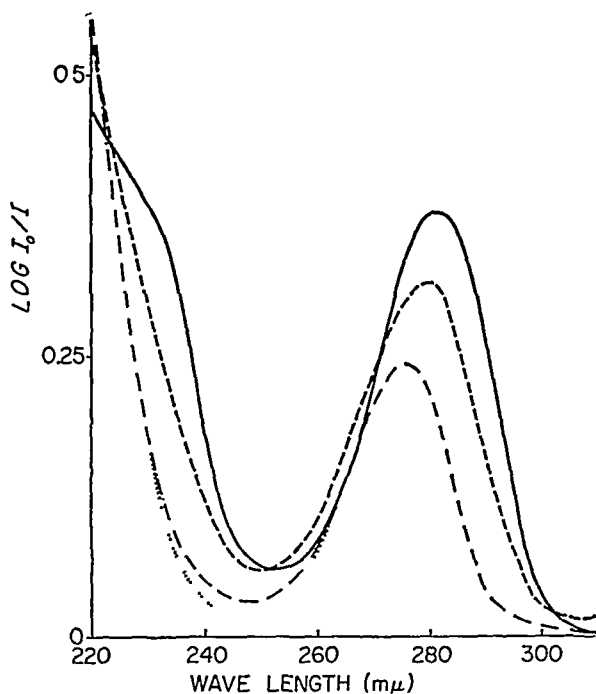


FIG 6

FIG 5 Effect of varying borate concentrations on the absorption spectrum of dopa (0.1 mm) at pH 7.8 —, zero borate, ---, 1.0 mm borate, - · -, 5.0 mm borate, - - -, 20 mm borate, - - - - , 40 mm borate

FIG 6 Effect of varying boric acid concentrations on the absorption spectrum of catechol (0.1 mm) —, values for zero borate at pH 5.7, - · -, for zero borate and pH 7.8, - - -, for 300 mm borate and pH 5.7, - - - - , for 50 mm borate and pH 7.8, the values for zero borate at pH 5.7 and 7.8 overlap below 230 mμ and above 270 mμ

that the free diphenol was the same in each case. The results are shown in Table II. The observed inhibition was calculated as $100(1 - v_i/v)$ where v_i is the velocity in the presence of inhibitor and v is the velocity in the absence of inhibitor. The calculated inhibition was obtained by using the K obtained from the absorption measurements of the complex.

The experiment shows that the velocity is nearly the same at the various diphenol and borate concentrations as that required by theory. Also, the observed and calculated inhibitions are within the experimental error, and

TABLE I
*Equilibrium Constant for Formation of Substrate-Borate Complex
 Obtained Spectrophotometrically at pH 7.8*

Compound	$K_{H_2BO_3}^*$	$K_{H_2BO_3}^{-\dagger}$	K_i^\ddagger
Catechol	600	1.9×10^4	5.3×10^{-5}
Dihydroxyphenylalanine	475	1.5×10^4	6.7×10^{-5}

* Equilibrium constant calculated by use of the H_2BO_3 concentration

† Equilibrium constant calculated by use of the $H_2BO_3^-$ concentration

‡ Equilibrium constant for the dissociation of the substrate-borate complex and equal to $1/K_{H_2BO_3}^-$

TABLE II
Correlation between Spectral and Enzymatic Data

Dopa	Borate	Velocity*	Velocity†	Free dopa	Complex	Per cent observed	Per cent inhibition calculated
mM	mM			mM	mM		
0.20	32.6	0.029	0.280	0.0125	0.188	90	94
0.10	15.2	0.027	0.161	0.0125	0.0875	83	86
0.08	11.7	0.030	0.128	0.0125	0.0675	77	81
0.06	8.3	0.031	0.103	0.0125	0.0475	68	70
0.04	4.8	0.030	0.075	0.0125	0.0275	61	63
0.02	1.3	0.029	0.043	0.0125	0.0075	33	38

The experiments were conducted at pH 7.8

* In the presence of borate expressed as the change in optical density at 305 $m\mu$ per minute

† In the absence of borate

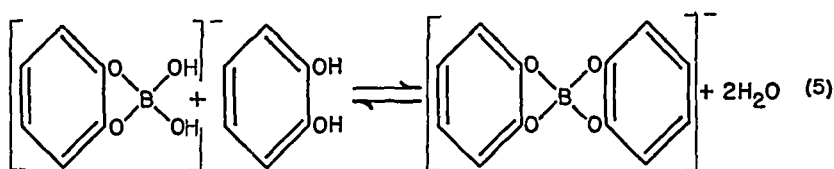
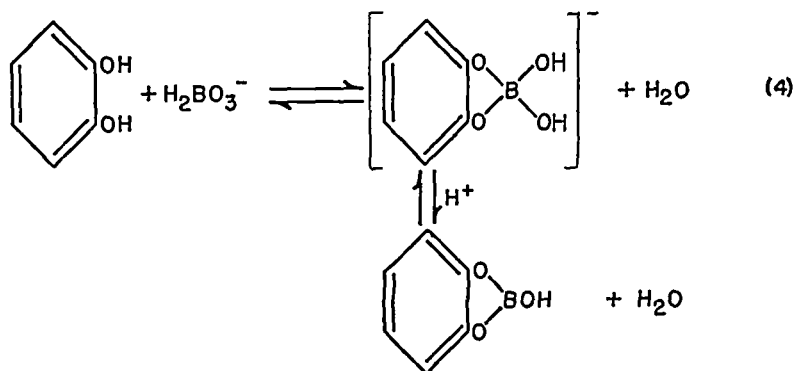
TABLE III
Enzymatically Obtained Dopa-Borate Dissociation Constants

pH	H_2BO_3	Calculated $H_2BO_3^-$ concentration	K_i
	mM	M	M
Dopa (265 $m\mu$ in presence of ascorbate), $K_m = 3.3 \times 10^{-4}$			
8	3.33	1.33×10^{-4}	5.1×10^{-5}
7	6.67	3.3×10^{-5}	5.0×10^{-5}
6	33.3	8.5×10^{-6}	4.2×10^{-5}
Dopa (305 $m\mu$ in absence of ascorbate), $K_m = 5.0 \times 10^{-4}$			
8	3.33	1.33×10^{-4}	6.9×10^{-5}
7	9.99	6.4×10^{-5}	6.7×10^{-5}
6	66.7	21.5×10^{-5}	6.4×10^{-5}

furthermore the correlation between the enzymatic data and the spectral data may be seen

Listed in Table III are the inhibitor-dissociation constants obtained from the $1/v$ versus $1/s$ plot. For the first set of data, the velocity was measured by the rate of oxidation of ascorbic acid at $265\text{ m}\mu$. In the second set of data, the change in density at $305\text{ m}\mu$, in the absence of ascorbic acid, was measured. If the reciprocals of the average K_i for the first set of data are taken, a value of 2×10^4 is obtained for the formation of the complex from dihydroxyphenylalanine and the H_2BO_3^- ion. In the second set of data, the equilibrium constant for the formation of the complex was 1.5×10^4 . These values are in general agreement with the equilibrium constant obtained from the spectral data.

Nature of Complex—Isbell *et al.* (8) have discussed the various complexes which are formed when borate is added to catechol, and the reactions are shown in the accompanying Equations 4 and 5. Schafer (9) has prepared



both the monocatechol-borate and the dicatechol-borate complexes. In our work, the ratio of borate to diol was much greater than that used by Schafer to prepare the monocatechol-borate complex, and therefore the concentration of the dicatechol-borate complex was negligible. In addition, Schafer found that the formation of the dicatechol-borate complex was slow and required several hours, whereas in the present work the solutions were tested immediately after the borate and diol were mixed. Furthermore, the agreement of the equilibrium constant obtained enzymatically and spectrophotometrically verifies the nature of the complex.

Reversal of Inhibition and Increased Absorption by Mannitol

Fig 7 demonstrates the reversal of both the inhibition and the increased absorption at pH 7.8 when mannitol is added to a solution containing 0.1 mM dihydroxyphenylalanine and 11.7 mM borate. There was a decrease

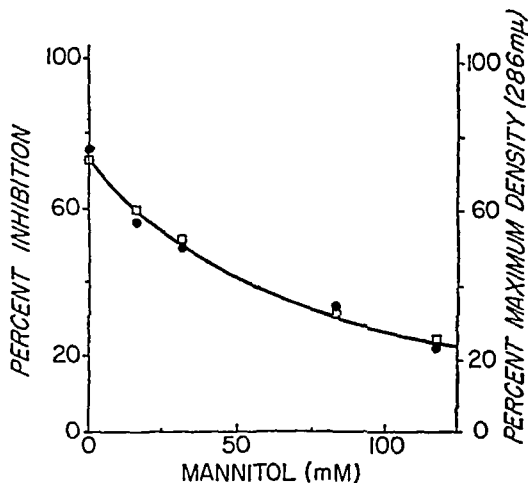


FIG 7 Reversal of borate inhibition and absorption changes by the addition of mannitol. In these experiments the dihydroxyphenylalanine concentration was 0.1 mM and the borate concentration was kept constant at 11.7 mM, the mannitol concentration was varied.

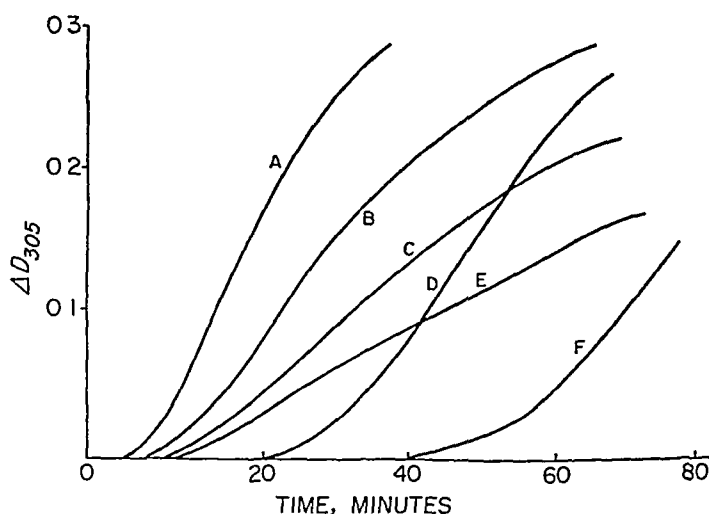


FIG 8 Effect of borate on tyrosine oxidation. The tyrosine concentration was kept constant at 0.04 mM. Curves A, D, and F were carried out at pH 7.8 with zero borate, 5.0 mM borate, and 10.0 mM borate, respectively. For Curves B, C, and E, the pH was 5.7 and the borate concentration was zero, 0.1 M, and 0.2 M, respectively.

in pH with higher concentrations of mannitol, but the change is insufficient to account for the reversal

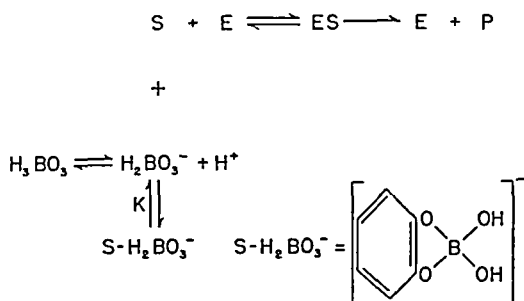
Effect of Borate on Oxidation of Monophenol

Behn and Nelson (10) noted that borates prolong the lag period in the oxidation of monophenols by tyrosinase at pH 8.5. We confirmed Nelson's finding of a prolonging of the lag period in alkaline solutions (Fig. 8) and found that the inhibition shown by the steepest part of the curves at pH 5.7 and 7.8 checks with the inhibition which might be expected from the assumption that it is due to the reaction of the H_2BO_3^- ion with the diphenol formed as an intermediate in the reaction.

The activity was measured as the change in optical density at 305 $\text{m}\mu$. In the presence of ascorbic acid and tyrosine, borate also inhibits the action of the enzyme.

SUMMARY

It has been concluded that borate inhibits tyrosinase, as shown in the accompanying equation



Evidence from enzyme studies and changes in the absorption spectrum of the diphenol when borate is added to buffered solutions of the diphenol are presented. Approximate equilibrium constants for the formation of the complex were calculated.

One of the authors (K. T. Y.) is greatly indebted to Dr. Walter B. Dandliker for his advice and encouragement upon the untimely death of Dr. E. R. Norris.

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INCORPORATION OF DEUTERIUM INTO OXIDIZED PYRIDINE NUCLEOTIDES BY ILLUMINATED GRANA*

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(Received for publication, January 21, 1957)

The reduction of pyridine nucleotides by illuminated chloroplasts was first demonstrated some 5 years ago by Vishniac and Ochoa (1, 2), Tolmach (3), and Ailon (4). In these experiments, the formation of reduced pyridine nucleotides was demonstrated indirectly by coupling the photochemical reaction with a suitable dehydrogenase and measuring the formation of the product of the dehydrogenase system. In the absence of the coupling system, no directly measurable reduction of pyridine nucleotides was observed (5). This inability of pyridine nucleotides to undergo directly measurable photochemical reduction was considered to be a consequence of their low oxidation-reduction potential¹ (E'_0 at pH 7 = -0.32 volt) since most substances which are effective as oxidants in the Hill reaction have high oxidation-reduction potentials (E'_0 at pH 7 = $+0.1$ to $+0.4$ volt).

In view of the physiological significance of the pyridine nucleotides as coenzymes in numerous enzyme-catalyzed reactions, it seemed worth while to investigate the mechanism of reduction of these compounds by illuminated grana. The experimental approach envisaged at the start of these studies was to determine the stereospecificity, if any, of the reduction of pyridine nucleotides by illuminated grana with deuterium as a tracer. It was hoped that the information obtained in these studies would permit us to determine whether the reduction was enzymatic or chemical in nature. If the pyridine nucleotide had remained unlabeled during alternate reduction and reoxidation by illuminated grana in heavy water, one could have concluded that both reduction and oxidation were enzymatic processes of identical stereospecificity.²

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¹ In 1952, the accepted E'_0 value for the DPN-DPNH potential was -0.28 volt. It has subsequently been shown to have a value of -0.32 volt by Burton and Wilson (6) and Rodkey (7).

² For a complete description of the stereospecificity exhibited by pyridine nucleotide-linked dehydrogenases, see the reviews by Vennesland (8) and San Pietro (9).

However, the data presented in this paper demonstrate that deuterium from the medium is incorporated into oxidized pyridine nucleotides by illuminated grana and that the deuterium is present at the site at which pyridine nucleotides undergo reversible oxidation-reduction, namely, at carbon atom 4 of the pyridine ring (10, 11). This result can be explained in terms of either a stereospecific enzymatic reduction or a non-stereospecific chemical reduction (see "Discussion"). The most likely explanation of these data involves a stereospecific reduction of the pyridine nucleotide, followed by a stereospecific oxidation of the reduced nucleotide, and the fact that the stereospecificities exhibited by the reduction and oxidation processes are opposite to each other. Evidence in support of this explanation has been presented by San Pietro and Lang (12), who have demonstrated the accumulation of reduced pyridine nucleotides by illuminated grana under certain conditions. In addition, recent experiments have provided more direct evidence that this reduction is indeed enzyme-catalyzed.³

Materials and Methods

The grana were prepared as follows. 25 gm of spinach leaves, purchased at a local grocery, were deveined and ground for 3 minutes in a Waring blender with 200 ml of cold 0.01 M $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 7, containing 0.01 M KCl. After being filtered through cheesecloth and glass wool, the filtrate was centrifuged for 2 minutes at $4600 \times g$. The supernatant fluid was centrifuged for 20 minutes at $18,000 \times g$, the residue was suspended in buffer and centrifuged again at $18,000 \times g$. The final residue was made up in 0.05 M $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 7, containing 0.01 M KCl, which was prepared in 99.5 per cent D_2O .⁴ The ability of the grana to reduce ferricyanide was determined before each deuterium experiment by measuring oxygen evolution in a Warburg apparatus modified for photosynthetic experiments.

Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) were purchased from the Pabst Laboratories.⁵ The DPNase was a purified enzyme which was obtained from zinc-deficient *Neurospora* according to the method of Kaplan *et al* (13). Crystalline alcohol dehydrogenase was purchased from the Worthington Biochemical Corporation.

The general experimental procedures used in these studies have been described in detail by Pullman *et al* (10), chlorophyll concentration was measured by the modification of Arnon (14), and deuterium content was

³ San Pietro, A., and Lang, H. M., unpublished data.

⁴ The heavy water used in these experiments was obtained on allocation from the United States Atomic Energy Commission.

⁵ Part of the DPN used in these experiments was a gift from the Pabst Laboratories.

determined by a modification of the method of Graff and Rittenberg (15, 16)

Results

Deuterium Incorporation into DPN—These experiments were set up in the following manner. The reaction mixtures, which contained DPN,

TABLE I

Incorporation of Deuterium into DPN by Illuminated Grana

Each reaction mixture contained grana equivalent to 14 mg of chlorophyll, 59 μ moles of DPN, 1000 μ moles of $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 7, and 200 μ moles of KCl. The final volume was 20 ml and the final concentration of D_2O was calculated to be about 90 per cent. The samples were incubated for 150 minutes either in the light or in the dark with shaking at 15–16°. The dark control flask was wrapped with tinfoil. The light reaction flasks were illuminated by one 100 watt bulb per flask at a distance of approximately 6 inches. In Experiment 6d, the gas phase was nitrogen rather than air. After incubation, the samples were centrifuged for 20 minutes at $18,000 \times g$ to remove the grana and the DPN concentration in the supernatant solution was determined by measuring the difference in optical density at 325 $m\mu$ in 1.0 M KCN, before and after the addition of *Neurospora* DPNase, according to the method of Colowick *et al.* (17). The recovery of DPN was almost quantitative. When all the DPN was split, the mixtures were placed on Dowex 1 formate columns. The nicotinamide was eluted with water, diluted by a known factor with unlabeled nicotinamide, and isolated by crystallization from benzene (10). The crystalline samples of nicotinamide were analyzed for deuterium content in the usual manner.

Experiment No	Conditions	Dilution factor	Deuterium content	
			Atom per cent excess*	Atom per molecule†
6a	Dark	9.3	0.03	0.02
6b	Light	9.5	0.87	0.50
6c	"	8.7	0.88	0.46
6d	Light-nitrogen	20.0	0.52	0.62

* Experimental values

† Values corrected for dilution by carrier-unlabeled nicotinamide. The deuterium content of the nicotinamide, expressed as atom of deuterium per molecule, is identical to the deuterium content, expressed similarly of the DPN from which it was derived (10). A deuterium content of 1 atom of deuterium per molecule corresponds to a value of 16.7 atom per cent excess for nicotinamide.

grana, buffer, and D_2O , were incubated either in the dark or in the light. In one experiment (Experiment 6d), the gas phase was nitrogen rather than air. After incubation, the grana were removed by centrifugation and the DPN was split with *Neurospora* DPNase. The resulting nicotinamide was isolated and analyzed for deuterium content.

The results of the isotope measurements are presented in Table I. It

can be seen that, when the reaction mixture was incubated in the dark, the DPN contained only 0.02 atom of deuterium per molecule (Experiment 6a) whereas, when the reaction mixture was illuminated, the DPN contained an average of 0.48 atom of deuterium per molecule (Experiments 6b and 6c). It is interesting that the DPN contained 0.62 atom of deuterium per molecule when the reaction mixture was incubated under nitrogen rather than aerobically (Experiment 6d). The explanation of this somewhat increased labeling is not readily apparent at the present time.⁶

These results do, however, clearly demonstrate that the incorporation of deuterium from the medium into DPN in the presence of grana is a

TABLE II

Incorporation of Deuterium into TPN by Illuminated Grana

Each reaction mixture contained grana equivalent to 16.1 mg of chlorophyll, 53.5 μ moles of TPN, 1000 μ moles of $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 7, and 200 μ moles of KCl. The final volume was 20 ml and the final concentration of D_2O was calculated to be approximately 82 per cent. The reaction mixtures were incubated for 120 minutes either in the light or in the dark, as described in Table I, with shaking at 15–16°. After incubation, the samples were treated as indicated in Table I. In each experiment, crystalline samples of nicotinamide were used for the deuterium analyses.

Experiment No	Conditions	Dilution factor	Deuterium content	
			Atom per cent excess*	Atom per molecule†
7a	Dark	12.7	0.03	0.02
7b	Light	12.1	0.62	0.45
7c	"	13.2	0.61	0.48

* Experimental values

† Values corrected for dilution by carrier-unlabeled nicotinamide

light-dependent reaction. In separate experiments it was demonstrated that the ability of illuminated grana to catalyze this incorporation of deuterium into DPN was completely lost by heating the grana for 5 minutes at 100°.

Deuterium Incorporation into TPN—The ability of illuminated grana to catalyze the incorporation of deuterium from the medium into TPN was determined in a manner similar to that described above for DPN. The results of the isotope measurements are presented in Table II. It can be

⁶ There was some reduced DPN present in this reaction mixture after incubation and removal of the grana. If the unlabeled DPN in the reaction mixture was reduced preferentially to the deuterium-labeled DPN during the incubation, i.e. if there was an isotope effect, then the remaining oxidized DPN would be enriched in deuterium. This could explain the increased labeling of the DPN remaining at the end of the reaction observed in this experiment.

seen that no deuterium is incorporated into TPN in the dark (Experiment 7a), whereas with illuminated grana the TPN remaining at the end of the reaction contained about one-half an atom of deuterium per molecule (Experiments 7b and 7c). These results are in excellent agreement with those presented in Table I for DPN.

Position of Deuterium in DPN—Having established that illuminated grana can catalyze the incorporation of deuterium into DPN from the

TABLE III

Site of Deuterium Incorporation into DPN by Illuminated Grana

The reaction mixture contained grana equivalent to 30.8 mg of chlorophyll, 121 μ moles of DPN, 2000 μ moles of $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 7, and 400 μ moles of KCl. The final volume was 40 ml and the final concentration of D_2O was calculated to be about 87 per cent. During incubation, the reaction mixture was divided into two 20 ml portions. Each portion was incubated for 120 minutes in the light, as indicated in Table I with shaking at 15–16°. After incubation, the grana were removed by centrifugation and the DPN was precipitated from the combined supernatant solution with acid-acetone and collected by centrifugation. The precipitate was dissolved in water and the solution, which contained 75 μ moles of DPN, was divided into two fractions. In Experiment 8a, the DPN present in one fraction was split with *Neurospora* DPNase, and the nicotinamide was isolated as indicated in Table I. In Experiment 8b the second fraction was treated with sodium dithionite (in H_2O) to yield chemically reduced DPN, which was isolated as the barium salt. The reduced DPN, as the sodium salt, was oxidized enzymatically with acetaldehyde and yeast ADH and the resulting DPN was treated as in Experiment 8a. In each experiment, crystalline samples of nicotinamide were used for the deuterium analyses.

Experiment No	Dilution factor	Deuterium content		Per cent retention of deuterium
		Atom per cent excess*	Atom per molecule†	
8a	20.4	0.36	0.44	75
8b	27.4	0.20	0.33	

* Experimental values

† Values corrected for dilution by carrier-unlabeled nicotinamide

medium, it was of interest to ascertain the site of the deuterium in the nucleotide. A sample of DPN which had incorporated deuterium as previously described was treated with sodium dithionite (in H_2O) to yield chemically reduced DPN, which was isolated as the barium salt. The reduced DPN, as the sodium salt, was oxidized enzymatically with acetaldehyde and yeast alcohol dehydrogenase and the deuterium content of the resulting oxidized DPN was determined. The isotope measurements for these experiments are presented in Table III. It can be seen (Experiment 8a) that the starting DPN contained 0.44 atom of deuterium per molecule. Chemical reduction followed by enzymatic oxidation (Experiment 8b)

yields DPN, which contains 0.33 atom of deuterium per molecule. One may conclude from these results that deuterium can be transferred by the procedure employed. The deuterium must, therefore, have been present at the site at which DPN undergoes reversible oxidation and reduction. Since it is known that the site of reversible oxidation and reduction is the γ position of the nicotinamide moiety of DPN (10, 11), it follows that the deuterium removed by reduction and reoxidation was present at this position.

The quantitative aspects of these experiments (Table III, last column)

TABLE IV
Effect of Length of Incubation on Incorporation of Deuterium into DPN by Illuminated Grana

Each reaction mixture contained grana equivalent to 13.5 mg of chlorophyll, 55 μ moles of DPN, 1000 μ moles of $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 7, and 200 μ moles of KCl. The final volume was 20 ml and the final concentration of D_2O was calculated to be about 86 per cent. The reaction mixtures were incubated for varying times in the light, as indicated in Table I, with shaking at 14–15°. After incubation, the samples were treated as described in Table I. In each case, crystalline samples of nicotinamide were used for the deuterium analyses.

Time of incubation	Dilution factor	Deuterium content	
		Atom per cent excess*	Atom per molecule†
<i>min</i>			
15	12.1	0.22	0.16
30	11.2	0.47	0.32
60	11.3	0.71	0.48
120	12.7	0.84	0.64

* Experimental values

† Values corrected for dilution by carrier-unlabeled nicotinamide

are in reasonable agreement with data previously published from this laboratory on the effects of oxidation and reduction on the deuterium content of γ -labeled DPN (18). One may conclude, therefore, that most or all of the deuterium found in DPN after illumination is in the γ position.

Deuterium Incorporation Versus Time—The rate of incorporation of deuterium into DPN by illuminated grana was determined as follows. Individual reaction mixtures, containing DPN, grana, buffer, and D_2O , were each incubated aerobically in the light. After incubation for a certain length of time, the grana were removed by centrifugation and the DPN was split with *Neurospora* DPNase. The resulting nicotinamide was isolated and analyzed for deuterium content.

The results of the isotope measurements are presented in Table IV. It

can be seen that the deuterium content of the oxidized DPN remaining at the end of the experiment increases with increasing time of incubation. It is clear from these data that the rate of incorporation of deuterium into DPN by illuminated grana in these experiments is linear for at least the first 30 minutes. After this time, the rate decreases as would be expected from the high degree of labeling in the nucleotide.

Effect of Grana Concentration—The effect of grana concentration on the incorporation of deuterium into DPN was determined in the following manner. Individual reaction mixtures, which contained the same amount

TABLE V
*Effect of Grana Concentration on Incorporation of Deuterium
into DPN by Illuminated Grana*

Each reaction mixture contained grana equivalent to varying amounts of chlorophyll, 63.5 μ moles of DPN, 1000 μ moles of $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 7, and 200 μ moles of KCl. The final volume was 20 ml and the final concentration of D_2O was calculated to be about 85 per cent. The reaction mixtures were incubated for 120 minutes in the light, as indicated in Table I, with shaking at 14–15°. After incubation, the samples were treated as described in Table I. In each case, crystalline samples of nicotinamide were used for the deuterium analyses.

Chlorophyll content	Dilution factor	Deuterium content	
		Atom per cent excess*	Atom per molecule†
mg			
3.6	9.2	0.35	0.19
7.2	10.1	0.68	0.41
10.9	9.9	0.79	0.47
13.6	11.8	0.77	0.55

* Experimental values

† Values corrected for dilution by carrier-unlabeled nicotinamide

of DPN, buffer, and D_2O but different amounts of grana, were incubated aerobically in the light. After incubation, the grana were removed by centrifugation. The DPN was split with *Neurospora* DPNase and the resulting nicotinamide was isolated and analyzed for deuterium content.

The results of the isotope measurements are presented in Table V. It is clear from these data that the amount of deuterium incorporated into DPN is dependent on the grana concentration. At low grana concentration, i.e. below a concentration equivalent to 7.2 mg of chlorophyll, there is a linear relationship between the grana concentration and degree of labeling of the DPN. At higher grana concentrations, there is no longer a linear relationship between the isotope content of the DPN and grana concentration.

DISCUSSION

The data presented above clearly indicate that deuterium from the medium is incorporated into DPN or TPN by illuminated grana. The intimate mechanism of this incorporation, however, is not clearly understood at the present time.

The most likely mechanism would involve reduction of the nucleotide followed by oxidation of the reduced nucleotide. In this manner, deuterium would be incorporated into the nucleotide *provided that the stereospecificities of the reduction and oxidation processes were not identical*.

If the stereospecificity of the reduction process was identical with that exhibited by the oxidation process, the oxidized nucleotide would never contain any deuterium. Under these conditions, the deuterium atom introduced into the reduced nucleotide in the reduction process would be removed upon subsequent oxidation of the reduced nucleotide. Hence, the oxidized nucleotide would remain unlabeled. This possibility may be eliminated therefore, since the data clearly indicate that deuterium is incorporated into DPN or TPN by illuminated grana.

The various types of stereospecificity which either the reduction or oxidation process may be expected to exhibit can be classified as follows: (a) stereospecific for side 1 of the pyridine ring of the nucleotide,⁷ (b) stereospecific for side 2 of the pyridine ring of the nucleotide, and (c) a lack of stereospecificity, *i.e.* non-stereospecific. The possible combinations of these types of stereospecificity which would result in the incorporation of deuterium from the medium into DPN or TPN are as follows: (1) the reduction process is stereospecific for side 1 and the oxidation process for side 2 or vice versa, (2) the reduction process must be stereospecific for either side 1 or side 2 and the oxidation process must be non-stereospecific, (3) the third possibility is merely the converse of (2), *i.e.* a non-stereospecific reduction followed by an oxidation which is stereospecific for either side 1 or side 2 of the pyridine ring, (4) lastly, it is possible that both the reduction and oxidation processes are non-stereospecific.

Any of these possibilities will result in the incorporation of deuterium from the medium into the oxidized pyridine nucleotide. It is, therefore, not possible to rule out any of them merely because of the deuterium data alone. However, in view of the evidence of San Pietro and Lang (12) that the reduction process is an enzyme-catalyzed reaction, it seems reasonable that possibilities (3) and (4) are rather unlikely ones. To date, every enzyme-catalyzed reaction which has been studied has been shown to be stereospecific for either side 1 or side 2 of the pyridine ring.² It would

⁷ Side 1 is defined here as that side of the pyridine ring to and from which hydrogen is transferred by yeast alcohol dehydrogenase (18, 19).

appear to be rather unlikely, therefore, that the reduction process under consideration, which is an enzyme-catalyzed reaction, would not also be stereospecific for either side 1 or side 2 of the pyridine ring

It is difficult to decide between possibilities (1) and (2). Any final decision must await the elucidation of the nature of the stereospecificity, if any, of the oxidation process which is operative in these studies

As mentioned previously, the rate of incorporation of deuterium into DPN by illuminated grana is linear for about the first 30 minutes (Table IV). This finding allows one to calculate the initial rate of reduction of DPN required to account for the degree of labeling observed in the DPN. (Since there was no accumulation of reduced DPN in these experiments, all of the DPN which was reduced was subsequently reoxidized.) In Table IV we see that, at the end of 15 minutes incubation, the DPN contained 0.16 atom of deuterium per molecule. Since the reaction mixture contained 55 μ moles of DPN, this means then that 0.16×55 or 8.8 μ atoms of deuterium had been incorporated in this time. Since the deuterium content of the medium was 86 per cent, then 0.86 μ atom of deuterium would be incorporated per micromole of nucleotide reduced and reoxidized, assuming the absence of an isotope effect and, further, that possibility (1) is the correct mechanism. Under these conditions, the deuterium atom incorporated into the reduced DPN upon reduction of unlabeled DPN would not be removed on subsequent oxidation and would appear in the oxidized nucleotide. Therefore, 8.8/0.86 or 10.2 μ moles of DPN must have undergone reduction (and subsequent reoxidation) in 15 minutes. The initial rate of nucleotide reduction is, therefore, $10.2 \times 4/135$ or 3 μ moles per hour per mg of chlorophyll.

If one assumes that possibility (2) is correct, and, in addition, also assumed the absence of an isotope effect, then the rate of nucleotide reduction would be higher than that calculated above, since less deuterium would be incorporated under these conditions per micromole of DPN reduced and subsequently reoxidized. In this case, as above, the same amount of deuterium would be incorporated in the reduction process. However, a part of the deuterium present in the reduced DPN would be removed if the oxidation process was not stereospecific. Thus, only a fraction of the deuterium present in the reduced DPN would appear in the oxidized DPN. Therefore, a greater amount of nucleotide would have to undergo reduction and subsequent oxidation to account for the amount of deuterium present in the oxidized nucleotide.

The rate of DPN reduction calculated above (3 μ moles per hour per mg of chlorophyll) is comparable to that reported by Vishniac and Ochoa (2) for the reduction of pyridine nucleotides in the presence of an added coupling system. However, it is quite low compared to the rate of photo-

chemical reduction of ferricyanide by the grana used in these experiments (12 μ moles per hour per mg of chlorophyll) and very much lower than the rate of photosynthesis in intact plants. It is interesting, however, that the rate of DPN reduction appears to be the same whether or not an external coupling system is provided. This finding supports the hypothesis proposed by Vishniac and Ochoa (2) that "the rate-limiting step in these experiments is probably the reduction of the pyridine nucleotides. The grana may be deficient in one or more of the factors required to transfer hydrogen from the photolytic system to the pyridine nucleotides." Additional evidence in support of this hypothesis has recently been obtained in our laboratory. We have been able to obtain a soluble extract from chloroplasts or whole spinach leaves which is required for the reduction of pyridine nucleotides under the conditions of low grana concentration. A description of the preparation and properties of this extract is in preparation.³

Faster rates of nucleotide reduction have been reported by Hendley and Conn (20), using the TPN-glutathione reductase system from wheat germ or parsley. It is possible that the reductase preparations used in these experiments contained the enzyme we have isolated from chloroplasts (or whole spinach leaves) which is required for the reduction of pyridine nucleotides as indicated above.

The authors are indebted to Dr. Sidney P. Colowick for many helpful suggestions and discussions and to Dr. Nathan O. Kaplan for a generous gift of DPNase.

SUMMARY

Experiments have been described which indicate that grana obtained from spinach leaves can incorporate deuterium from the medium into di- or triphosphopyridine nucleotide in the light. A possible mechanism for this incorporation is discussed.

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STUDIES ON THE DISSOCIATION OF HISTONES FROM THE NUCLEOHISTONE OF CALF THYMUS*

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Complexes which contain deoxyribonucleic acids and histones have been extracted from a wide variety of tissues (2) Mirsky and Ris (3) have demonstrated that the components of such complexes occur combined in isolated chromosomes. However, nucleohistone complexes can also be produced *in vitro* simply by mixing solutions of the constituent polyelectrolytes. For this reason, it is difficult to decide whether the products that can be isolated directly from tissues correspond to compounds which exist in the living cells or whether they are artifacts which arise during the extraction procedures. This problem, which has repeatedly received attention since the time of Kossel, has been discussed by a number of authors (Kossel (4), Mirsky and Ris (3), and Chargaff (5)).

Recent evidence has made it clear that both of the principal moieties of nucleohistones, the deoxyribonucleic acid (6-8) and the histone (see Crampton *et al* (9) for references in the literature) consist of mixtures of chemically different components. If these heterogeneous polyacids and polybases are already combined *in vivo*, it would not be unreasonable to expect that particular fractions of deoxyribonucleic acid are preferentially associated with particular histone fractions. The existence of specific combinations might be detected by partially dissociating a "native" nucleohistone on the one hand and, on the other hand, a "reassociated" nucleohistone which had been produced in the laboratory by recombining the separate constituents. The liberation under identical conditions of different products from the two types of nucleohistone would suggest that the fractions were originally associated in a specific manner. Previous studies revealed that fractions of deoxyribonucleic acid, which had similar base compositions,

* A brief description of portions of this work was presented before the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, April, 1956 (1).

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¹ The term "native" is not used in an absolute sense, but only in contrast to the term "reassociated." See Crampton *et al* (10) for a description of native and reassociated nucleohistones which were prepared by procedures slightly different from those employed here.

were liberated when native and reassociated nucleohistones were partially dissociated (8). In the present study the histones have been investigated. For this purpose, chromatography on IRC-50 (9) has been employed to characterize the mixtures of histones liberated.

EXPERIMENTAL

Unless otherwise noted, all operations were carried out at 4°. The native¹ nucleohistones (Experiments 1, 2, 5, and 6, Tables I and II) that served as the starting products for all further procedures were prepared from nucleohistone extracts obtained by 1 to 2 hours of extraction of washed residue of calf thymus with distilled water in the manner already described (9, 11). The reprecipitated native and reassociated products were prepared from these materials and subsequently processed in parallel to avoid the effects of differential aging. The reassociated products were prepared by briefly exposing the native nucleohistone to the dissociating action of strong neutral salt solutions (10). For reasons stated below, either NaCl or Ba(OAc)₂ was used for this purpose. Histone samples were ready for chromatography about 15 hours after the death of the animal. In order to determine their total ninhydrin color, the nucleohistone preparations were dissolved in suitable volumes of 0.6 M Ba(OAc)₂, and 1 ml aliquots were analyzed by the ninhydrin method.

Reassociated and Native Nucleohistones Prepared by Action of NaCl—To prepare reassociated nucleohistone, the native nucleohistone mentioned above was first suspended in distilled water (the product from 42 gm of calf thymus per 100 ml) and an equal volume of 4 M NaCl was added with thorough mixing. After 30 minutes, the viscous mass was slowly added, with gentle stirring, to a 9-fold volume of distilled water. The NaCl molarity was thereby lowered to 0.2 M. After 30 minutes, the fibrous precipitate of reassociated nucleohistone was collected and squeezed on a sintered glass funnel with a spatula to remove excess fluid.

To effect fractional dissociation of the histones from this reassociated nucleohistone (Experiments 7b and 8b, Table II), the product was first cut into small pieces and dispersed in distilled water (100 ml per 20 gm of original calf thymus) with the aid of a high speed mixer (see Crampton *et al* (11)). A 2-fold volume of 0.3 M Ba(OAc)₂ at pH 8 was added dropwise with mechanical stirring in order to bring the molarity to 0.2 M. When a different final concentration of Ba(OAc)₂ was desired, the molarity of the Ba(OAc)₂ solution added was adjusted accordingly. After 30 minutes, 2 volumes of ethanol were added, and 15 minutes later the mixtures were centrifuged (5 minutes at 2000 × g). The supernatant fluids were filtered, evaporated to dryness, redissolved, and adjusted for chromatography to 0.1 M Ba(OAc)₂, pH 6.

TABLE I
Dissociation of Nucleohistones by Aqueous Barium Acetate

Experiment No *	Initial nucleohistone Preparation No	Specimen of nucleohistone	Per cent ninhydrin color† liberated when final Ba(OAc) molarity was		
			0.10 M	0.15 M	0.20 M
1	22	Native, starting product			28
2	27	" " " ‡			32
3a	23	" Ba(OAc) ₂ -treated			25
3b		Reassociated, Ba(OAc) ₂ -treated			25
4a	26	Native, Ba(OAc) ₂ -treated§	11	27	32
4b		Reassociated, Ba(OAc) ₂ -treated	10	25	27

* Consult the experimental section of the text for details of the experimental arrangement

† Referred to the color given by the nucleohistone in aqueous 0.6 M Ba(OAc)₂

‡ In this experiment, the 0.3 M Ba(OAc)₂ added to the nucleohistone was pH 6 rather than pH 8

§ At 0.01 M and 0.06 M Ba(OAc)₂, pH 6, 0.4 and 3.5 per cent, respectively, of the ninhydrin color of this specimen remained in the supernatant fluid

TABLE II
Dissociation of Nucleohistones in Ethanolic Barium Acetate

Experiment No *	Initial nucleohistone Preparation No	Specimen of nucleohistone	Per cent ninhydrin color† liberated when final Ba(OAc) molarity was			
			0.05 M	0.067 M	0.20 M	0.33 M
5	21	Native, starting product		17		
		" " " ‡		18		
6	22	" " "			66	
7a	19	Native, NaCl-treated	13	20	72	
7b		Reassociated, NaCl-treated	11	11	68	
8a	20	Native, NaCl-treated	12	19		79
8b		Reassociated, NaCl-treated	11	17		81
9a	27	Native, Ba(OAc) ₂ -treated		21		
9b		Reassociated, Ba(OAc) ₂ -treated		30		

* Consult the experimental section of the text for details of the experimental arrangement

† Referred to the color given by the nucleohistone in aqueous 0.6 M Ba(OAc)₂

‡ In this experiment, the 0.3 M Ba(OAc)₂ added to the nucleohistone was pH 6 rather than pH 8

A reprecipitated native nucleohistone with an experimental history closely comparable to that of the reassociated product was prepared from the native nucleohistone starting material by adjusting the NaCl concen-

tration directly to 0.2 M as follows. A suspension of the native product in distilled water (the same concentration as was used to prepare the reassociated product) was thoroughly mixed with an equal volume of distilled water. After 30 minutes, the mixture was poured slowly with stirring into a 9-fold volume of 0.222 M NaCl. After an additional 30 minutes, the precipitate (largely granular) was collected by centrifugation. Fractional dissociation of this reprecipitated native nucleohistone was carried out with $\text{Ba}(\text{OAc})_2$ and ethanol, exactly as described above for the reassociated material (see Experiments 7a and 8a, Table II).

For purposes of comparison, the original native nucleohistone starting material (Experiments 5 and 6) was also fractionally dissociated in the manner described above. In some instances (Experiments 1 and 2), the histones present in the aqueous extract before the addition of ethanol were also determined by chromatographic analysis of an aliquot of the solution. In these experiments, the 0.2 M $\text{Ba}(\text{OAc})_2$ suspension of the nucleohistone (pH 8 in Experiment 1, and pH 6 in Experiment 2) was centrifuged, and the supernatant fluid was filtered, diluted to 0.1 M $\text{Ba}(\text{OAc})_2$, and adjusted to pH 6 (if necessary) before chromatography. The $\text{Ba}(\text{OAc})_2$ -ethanol extracts (Experiments 5 and 6) were obtained in exactly the manner described for Experiments 7a and 8a.

Reassociated and Native Nucleohistones Prepared by Action of $\text{Ba}(\text{OAc})_2$ —Differences between the reassociated nucleohistones and reprecipitated native nucleohistones prepared in NaCl might arise from unequal losses of histone to the supernatant fluids remaining after the final reprecipitation from 0.2 M NaCl. To cover this eventuality, reassociated and native nucleohistones, analogous to the products described in the previous section, were prepared by the use of $\text{Ba}(\text{OAc})_2$. In this manner, it was not necessary to discard the supernatant fluids before chromatography.

In Experiment 3a, the native nucleohistone starting product from 30 gm of calf thymus was dispersed in 100 ml of water. To one-half of this suspension, 21.5 ml of 2 M $\text{Ba}(\text{OAc})_2$ (pH 8.5) were added to increase the $\text{Ba}(\text{OAc})_2$ concentration to 0.6 M in order to promote dissociation. To bring about reassociation, 145 ml of water were added (after 30 minutes) and the resulting mixture (0.2 M in respect to $\text{Ba}(\text{OAc})_2$) was stirred for 30 minutes and centrifuged. The supernatant fluid was filtered, diluted to 0.1 M $\text{Ba}(\text{OAc})_2$ and adjusted to pH 6, and used directly for chromatography. The other half of the suspension of the native nucleohistone was diluted with 21.5 ml of water, followed by 145 ml of 0.3 M $\text{Ba}(\text{OAc})_2$ (pH 8) added slowly and with thorough mixing. After 30 minutes, the mixture was centrifuged as before, the supernatant fluid was filtered, diluted to 0.1 M $\text{Ba}(\text{OAc})_2$, adjusted to pH 6, and used directly for chromatography. This material is referred to as native nucleohistone ($\text{Ba}(\text{OAc})_2$ -treated) in Table I, Experiment 3a.

In Experiment 4b, three aliquots of a suspension of the original native nucleohistone starting product were diluted with an equal volume of 1 M $\text{Ba}(\text{OAc})_2$, pH 6, to promote dissociation. After 30 minutes, a sufficient volume of distilled water was added to the aliquots to reduce the molarity of $\text{Ba}(\text{OAc})_2$ from 0.5 to 0.2 M, 0.15 M, and 0.1 M, respectively. After 30 minutes the mixtures were centrifuged, and the supernatant fluids were filtered and diluted to 0.1 M $\text{Ba}(\text{OAc})_2$ for chromatography. The corresponding extracts of native nucleohistones (Experiment 4a) were prepared by diluting aliquots of the same suspension of native nucleohistone with an equal volume of distilled water. After 30 minutes, enough 0.33 M, 0.215 M, or 0.125 M $\text{Ba}(\text{OAc})_2$, pH 6, was added to raise the $\text{Ba}(\text{OAc})_2$ molarity from 0 to 0.2 M, 0.15 M, and 0.1 M, respectively. The mixtures were clarified and prepared for chromatography as above.

In Experiment 9b, native nucleohistone was dispersed in distilled water as before, and dissociation was effected by adding enough 1 M $\text{Ba}(\text{OAc})_2$, pH 6, to bring the mixture to 0.6 M. After 15 minutes, reassociation was allowed to take place by adding distilled water to reduce the concentration of $\text{Ba}(\text{OAc})_2$ to 0.2 M. After an additional 15 minutes, a 2-fold volume of 95 per cent ethanol was added. The mixture was centrifuged, and the supernatant fluid clarified, freed from alcohol, and prepared for chromatography as described for Experiments 7 and 8.

In the parallel Experiment 9a, native nucleohistone was partially dissociated by adding enough 0.231 M $\text{Ba}(\text{OAc})_2$ to a distilled water suspension of the histone to bring the concentration of $\text{Ba}(\text{OAc})_2$ to 0.2 M. Ethanol was then added and the mixture clarified and prepared for chromatography as for the reassociated product.

Chromatography of Histones—Appropriate aliquots of all samples of histones were chromatographed on 10×0.9 cm columns of Ba IRC-50 which had been equilibrated with 0.1 M $\text{Ba}(\text{OAc})_2$, pH 6. The eluent of gradually increasing $\text{Ba}(\text{OAc})_2$ concentration was produced by continually adding 1.0 M $\text{Ba}(\text{OAc})_2$, pH 6, to a 50 ml mixing chamber initially filled with 0.1 M $\text{Ba}(\text{OAc})_2$, pH 6 (see Crampton *et al* (11)).

Results

Dissociation of Nucleohistones by Aqueous $\text{Ba}(\text{OAc})_2$ —When native nucleohistone is suspended in 0.20 M $\text{Ba}(\text{OAc})_2$, a portion of the histone is brought into solution, but the entire nucleic acid remains insoluble. Chromatography of the products dissociated in this manner (from Experiments 1 and 2) reveals that only material emerging at the position of histone Fraction A is liberated (11). The fact that this fraction is readily dissociated from nucleic acid was exploited successfully in the methods used by Davison and Butler (12), by Davison and Shooter (13), and by Daly and Mirsky (14), who originally prepared materials that had amino acid com-

positions similar to the Fraction A subsequently isolated chromatographically (9, 11). From Table I it is apparent that both native and reassociated nucleohistones yield similar percentages of the total ninhydrin color when they are dissociated by the action of aqueous 0.2 M $\text{Ba}(\text{OAc})_2$ (Experiment 3, Table I), or by aqueous $\text{Ba}(\text{OAc})_2$ of lower molarity (Experiment 4). The chromatograms of the histones liberated are given in Fig 1, and show that only Fraction A is dissociated from both types of nucleohistone. Chromatograms (not shown) of the histones dissociated by aqueous $\text{Ba}(\text{OAc})_2$ at lower molarity also revealed that only histones emerging at the position of Fraction A were liberated. Attempts to dissociate Fraction B near neutrality by treating the nucleohistones with $\text{Ba}(\text{OAc})_2$

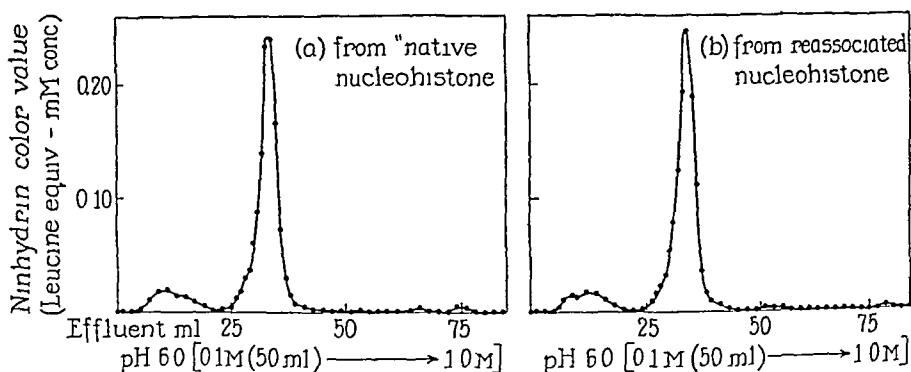


FIG 1 Chromatography of histones dissociated (a) from native, and (b) from reassociated, nucleohistones by 0.2 M $\text{Ba}(\text{OAc})_2$ (Experiment 3, Table I). In each experiment, a 6 ml sample, containing the product from 0.18 gm of calf thymus, was chromatographed.

at higher molarity were unsuccessful. Above 0.3 M $\text{Ba}(\text{OAc})_2$, nucleic acid begins to dissolve.

Thus, native and reassociated nucleohistones could not be distinguished by the amounts nor by the chromatographic behavior of the histones liberated by treatment with aqueous $\text{Ba}(\text{OAc})_2$.

Dissociation of Nucleohistones by $\text{Ba}(\text{OAc})_2$ and Ethanol—Whereas dilute aqueous $\text{Ba}(\text{OAc})_2$ is able to liberate substantially all of histone Fraction A and none of Fraction B, the action of $\text{Ba}(\text{OAc})_2$ and ethanol is much more complicated. In the first place, when a 2-fold volume of ethanol is added to nucleohistone dissolved in 0.6 M $\text{Ba}(\text{OAc})_2$, thereby lowering the Ba^{++} concentration to 0.2 M, the amount of ninhydrin color remaining in the supernatant fluid is twice as great as that found when a similar quantity of nucleohistone is exposed to 0.2 M aqueous $\text{Ba}(\text{OAc})_2$ (compare Experiments 1 and 2 with Experiment 6, Table II). This observation formed the basis of the more rapid procedures for the preparation of the histone mixtures from which Fractions A and B were isolated chromatographically.

(11) Chromatographic analysis of the $\text{Ba}(\text{OAc})_2$ -ethanol supernatant fluid reveals, as might be expected from the increased ninhydrin color, the presence of considerable Fraction B, in addition to Fraction A (Fig 2). The amount of Fraction A remaining in the ethanolic supernatant fluids, however, is less than would have been found in the aqueous $\text{Ba}(\text{OAc})_2$ extracts before the ethanol was added. Apparently ethanol, while liberating some of Fraction B, causes some of Fraction A to reassociate with the suspended nucleohistone.

Although native and reassociated nucleohistones cannot be differentiated by the histones liberated by aqueous $\text{Ba}(\text{OAc})_2$, a difference between the two preparations does appear when ethanol is added to the aqueous mixtures. This fact is illustrated in Figs 2 and 3. Fig 2 compares nucleohistones previously treated with NaCl , Fig 3 compares those previously treated with $\text{Ba}(\text{OAc})_2$. In Fig 2, it will be noted that at the lower concentrations of $\text{Ba}(\text{OAc})_2$, upon the addition of ethanol, more Fraction B is liberated and less Fraction A remains dissociated from the native than from the reassociated nucleohistone. Thus, in Fig 2, *a*, 9 per cent of the maximal obtainable Fraction A and 12 per cent of Fraction B are found, whereas the corresponding values for Fig 2, *d* are 20 and 6 per cent, respectively. If the final $\text{Ba}(\text{OAc})_2$ molarity is raised from 0.05 M (Fig 2, *a* and *d*) to 0.067 M (Fig 2, *b* and *e*), 35 per cent of Fraction A and 17 per cent of Fraction B are obtained from the native nucleohistone, and 44 per cent of Fraction A and 8 per cent of Fraction B from the reassociated material. A similar result is shown in Fig 3. However, the mixtures of histones obtained from the two types of nucleohistones when the final $\text{Ba}(\text{OAc})_2$ concentration is raised to 0.33 M are indistinguishable (Fig 2, *c* and *f*). Apparently the mixtures of histones obtained from native and reassociated nucleohistones by the action of $\text{Ba}(\text{OAc})_2$ and ethanol are not demonstrably different when more than about 30 per cent of the total histone has been liberated. The series of chromatograms (not shown) obtained from Experiment 7, Table II, was similar to those shown in Fig 2, which were obtained from Experiment 8.

Because it contains more arginine, histone Fraction B is more basic than histone Fraction A. This fact explains why Fraction B is eluted from the acidic resin IRC-50 less readily than histone Fraction A (9, 11). The partial dissociation experiments indicate that in native nucleohistone a significant portion of the more basic histone Fraction B is attached by linkages which are relatively easily ruptured by $\text{Ba}(\text{OAc})_2$ -ethanol. In reassociated nucleohistone, a much lower proportion of the histone Fraction B is easily liberated by $\text{Ba}(\text{OAc})_2$ -ethanol. It would therefore appear that some of the linkages initially present are not restored if native nucleohistone is dissociated by strong salt solutions and allowed to reassociate in dilute salt solution.

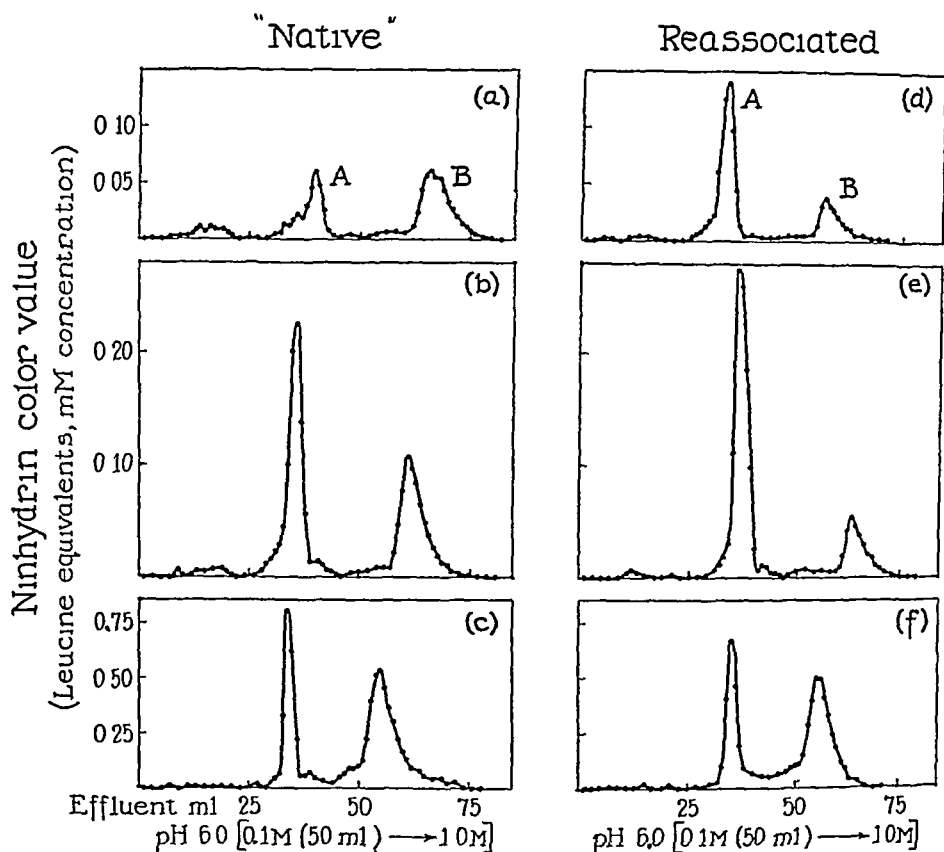


FIG 2 Chromatography of histones obtained from native (a, b, and c), and from reassociated (d, e, and f), nucleohistones by the addition of 2 volumes of ethanol to mixtures in 0.5 M $\text{Ba}(\text{OAc})_2$ (a and d), 0.20 M $\text{Ba}(\text{OAc})_2$ (b and e), and 0.10 M $\text{Ba}(\text{OAc})_2$ (c and f) (Experiment 8, Table II). Each pattern has been drawn to represent the product obtained from 0.4 gm of calf thymus.

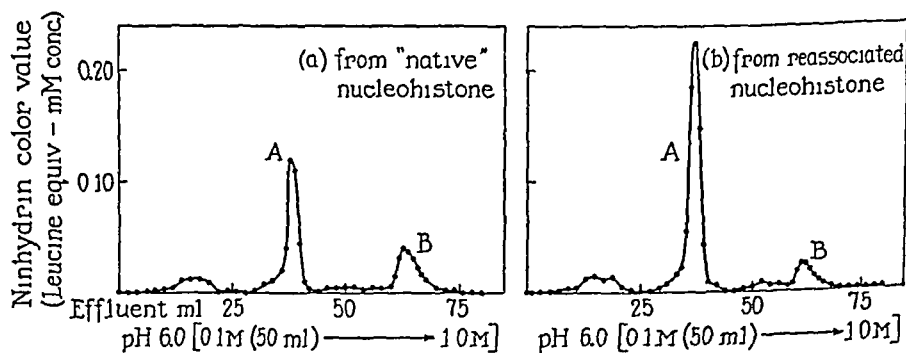


FIG 3 Chromatography of histones obtained (a) from native, and (b) from reassociated, nucleohistones by the addition of 2 volumes of ethanol to suspensions of the materials in 0.2 M $\text{Ba}(\text{OAc})_2$, the samples correspond to Experiment 9, Table II. In each experiment, an 8 ml sample, containing the product from 0.16 gm of calf thymus, was chromatographed.

The observed differences might depend on a number of physical or chemical features of the nucleohistones. If histones become associated with nucleic acids *in vivo* in a highly stereospecific manner, such as that proposed for the attachment of protamines to nucleic acid (15), the original mode of combination might not be restored upon more or less random reassociation *in vitro*². It is significant in this connection that the nucleohistone of calf thymus contains nearly as many residues of arginine plus lysine plus histidine as total residues of phosphorus³ (see Crampton *et al* (11), Table IV). The nature of the difference which has been detected in the present study would suggest that in the native nucleohistone a larger proportion of the more basic histone Fraction B is associated with less acidic fractions of the deoxyribonucleic acid than is the case in the reassociated nucleohistone. It is also possible that, in the native complex, the histones are bound to the nucleic acids principally by electrostatic forces, whereas in the reassociated product the histones are bound as well by non-ionic forces similar to those which cause isolated histones to aggregate⁴ with each other. Regardless of what factors ultimately are responsible for the differences, the present findings suggest that at least a portion of the components of nucleohistones are originally combined *in vivo* in a manner which is not restored upon random reassociation. In assuming a molecular weight of 10^6 for deoxyribonucleic acid (17) and a molecular weight of 10^4 for the histones (compare the discussion of the molecular weights of histones (11)), it is apparent that in the nucleohistone of calf thymus there must exist about 150 molecules of histone for each molecule of nucleic acid. Therefore, even within a single complex of average over-all composition, enormous variety could be achieved by varying the locations where the different protein molecules were joined to a molecule of nucleic acid. The possibility of the existence of such isomerism in the nucleoprotamines has been discussed by Felix and coworkers (18, 19). Dissociation and reassociation *in vitro* of the nucleohistones of calf thymus probably produce isomers in which the histone molecules have assumed new positions along the nucleic acid molecules. That many of the properties of nucleohistones would not be affected by such isomerism is indicated by previous work (20, 10, 8, 21), which showed that native and reassociated nucleohistones are indistinguishable according to certain criteria.

² Reassociation of the components at higher dilution might give a product more similar to the original, native nucleohistone. The properties of reassociated protamine nucleate appear to be markedly dependent upon the concentration of the protamine and nucleic acid in the solutions that are mixed (16).

³ Dr J A V Butler and Dr P F Davison (personal communication) have also noted a correlation between the number of arginine residues and the number of phosphate groups in the nucleohistone of calf thymus.

⁴ Trautman, R, and Crampton, C F, to be published.

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SUMMARY

Nucleohistone specimens which were prepared by conditions not conducive to dissociation of the component histones and deoxyribonucleic acids have been compared with the corresponding materials produced by intentional dissociation and reassociation. Treatment with $\text{Ba}(\text{OAc})_2$ and subsequently by ethanol under suboptimal conditions was used to liberate the histones from both types of nucleohistone. The histones liberated were determined by chromatography on Ba IRC-50. It was found that more histone Fraction B and less histone Fraction A remained dissociated after treatment of the native nucleohistone than was the case with the reassociated product. This finding has been interpreted to indicate that the original combination of histones and deoxyribonucleic acid *in vivo* is, at least in part, specific.

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CHROMATOGRAPHIC SEPARATION OF UROPORPHYRINS I AND III AND THE NATURE OF WALDENSTRÖM PORPHYRIN*

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PLATE 1

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Patients with porphyria usually discharge large amounts of uroporphyrins, mainly as a mixture of the isomers I and III, as well as other porphyrins. Knowledge concerning their quantitative distribution in the disease has long been sought. The chromatographic separation of methyl esters of uroporphyrin I and "Waldenstrom porphyrin" (1, 2), then believed to be uroporphyrin III, was described by Fischer and Hofmann (3), with talc as the adsorbent. Working with the same adsorbent, Grinstein, Schwartz, and Watson (4) and Watson, Schwartz, and Hawkinson (5) found no evidence of uroporphyrin III in the Waldenstrom porphyrin. Nicholas (6) also reported no separation of the isomers on talc and other adsorbents. The recent articles by Nicholas and Rimington (7), Kennard and Rimington (8), Watson and Berg (9), and Watson *et al* (10), while presenting the different views on the controversial Waldenstrom porphyrin, have indicated an urgent need of a better method of separating the uroporphyrin isomers. The present paper is concerned with the chromatographic separation of methyl esters of uroporphyrins I and III on a "Hyflo Super-Cel" column and the chemical nature of the so called Waldenstrom porphyrin isolated from acute cutanea, tarda and patients with a mixed type porphyria.

Procedure and Results

Materials

Uroporphyrin I (UI) methyl ester, m p 293–295°, was a gift from Dr Watson and Dr Schwartz,¹ uroporphyrin III (UIII) methyl ester (tardacin), m p 258–260°, was a gift from Dr Rimington,¹ and Gray's Walden-

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¹ The authors wish to thank Dr C J Watson and Dr S Schwartz of the University of Minnesota, Minneapolis, Dr C Rimington of the University College Hospital Medical School, and Dr C H Gray of King's College Hospital Medical School London, England, for their gifts of special porphyrins.

strom porphyrin ester, m p 251–256°, a typical sample from a patient with acute porphyria (11), was a present from Dr Gray¹

Other porphyrin samples were isolated from urine of patients with porphyria² Patient 2, a female aged 51, was afflicted with porphyria cutanea tarda for years but suffered no abdominal pain or nervous symptoms, Patient 3, a male aged 50, had the same symptoms as Patient 2, Patient 4, a male aged 52, a familial victim of intermittent acute porphyria, was totally paralyzed for 3 months, Patient 5, a female aged 43, was afflicted with a mixed type with abdominal pain and skin lesions, Patient 6, the son of Patient 5, aged 20, showed cutaneous lesions 6 months previously, Patient 7, an 81 year old male with cutanea tarda, suffered from sclerosis and severe skin lesion After his death from a brain hemorrhage, samples of the liver and serum were kept in a freezer

The total porphyrins were generally isolated from the urine samples by adsorption on talc, and from the liver by ethyl acetate-acetic acid and dilute ammonia extraction, followed by esterification and chromatography A number of less known porphyrins, including the one hereafter tentatively designated as "7III" porphyrin, its methyl ester melting at 217–218°, were isolated from large volumes of urine from Patients 2 and 3

The Waldenstrom porphyrin (1, 2) was prepared from the ether-extracted urine samples by extraction with ethyl acetate at pH 3.0 to 3.2 The ethyl acetate solution of the porphyrin was evaporated to dryness under a reduced pressure and esterified as usual

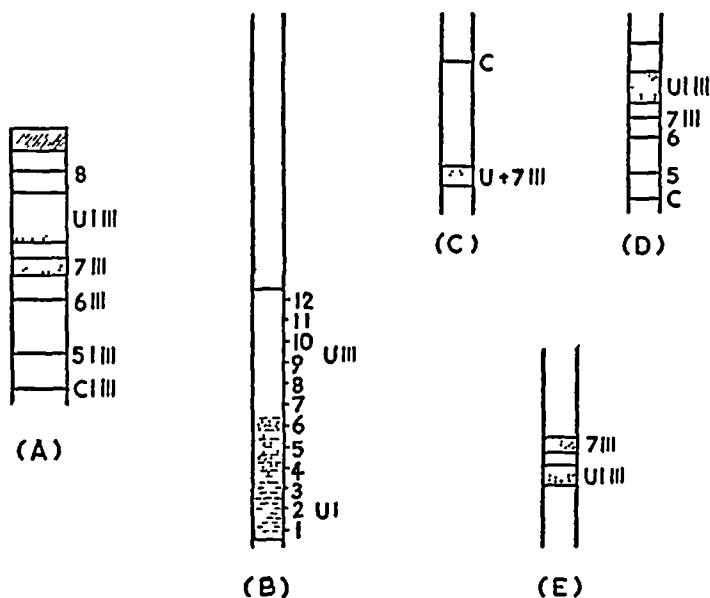
Chromatography

Hyflo Super-Cel Column Chromatography—The Hyflo Super-Cel is an amorphous diatomaceous silica produced by Johns-Manville Because of the loose and sandy nature of the adsorbent, the column was packed with small portions at a time of the dry material, with increasing suction The sample to be chromatographed was dissolved in a small volume of chloroform and mixed thoroughly with a small amount of Hyflo Super-Cel After the adsorbed sample was dried in air, it was carefully placed on the top of the column and packed to a uniform layer of 2 to 3 mm thickness Then another 1 cm layer of Hyflo Super-Cel was packed on top of the sample to protect it from being disturbed by the developing solvent and to maintain a uniform start of the development A slight suction, applied before the developing solvent was introduced, was maintained during the development and stopped as soon as the solvent front reached almost to

² Urine samples from Patients 2 and 3 were made available to us through the courtesy of Dr M Zimmerman of Whittier, California, and those from Patients 4 to 7 were provided through the courtesy of Dr A Redeker, Dr R Sterling, and Dr N Chiamori of the County Hospital, Los Angeles, California

the bottom of the column. With these precautions, streaking was prevented.

The developing solvent, used for the primary separation of the crude porphyrin esters, is composed of chloroform (U S P) and light petroleum ether (b p 30–60°) in a volume ratio of 1:2. The crude porphyrins from the cutanea tarda and mixed type patients (Patients 2, 3, 5, and 6) revealed



TEXT-FIG 1 Chromatograms of porphyrin methyl esters from porphyria urine. *A*, separation of the total porphyrins on the Hyflo column. C I III stands for coproporphyrin I and III, U I III for uroporphyrins I and III, 7 III, 5 I III, 6 III, and 8 for less known porphyrins. *B*, distribution of UI and UIII on a typical long Hyflo column. *C* and *D*, separation of the Waldenstrom porphyrin from an acute case (Patient 4) on an MgO column (6) and a Hyflo column, respectively, and *E*, separation of the Waldenstrom porphyrin from Patient 6 on an MgO column with a modified developing solvent (see the text).

more than six distinct zones on the column, representing more than eight different porphyrins. The major zone contained esters of uroporphyrins and was located just above the "7III" porphyrin zone (Text-fig 1, *A*). The zones were, respectively, cut out and eluted with chloroform. The isomers were not separated on the primary column.

For the separation of esters of uroporphyrins I and III, a longer chromatographic tube, 18 × 40 cm, was used. It was packed with Hyflo Super-Cel to a height of 30 cm or more. A thin layer of the sample-adsorbed Hyflo Super-Cel and another layer of plain Hyflo Super-Cel were successively introduced onto the top of the column as described before.

The column was first irrigated with 50 ml of benzene and then with 50 ml of a mixture of chloroform and benzene (both reagent grade, 1:2). It was followed successively by 50 ml portions of the same mixture containing, respectively, 1, 2, and 3 drops of ethyl alcohol (60 drops per ml). In about 1.5 hours the porphyrin front almost reached the bottom of the column. The volume of the last portion of the developer, that is the chloroform-benzene mixture containing 3 drops of alcohol per 50 ml, may be increased or reduced according to the location of the advancing porphyrin front. With the aid of ultraviolet light, the entire fluorescent zone, which might measure about 12 cm, was marked out in cm, and the fractions were removed cm by cm and dried in air. Each fraction was then repacked in a small Allihn type filter tube and eluted with chloroform. Of the dozen fractions, several lower ones contained only UI ester, the upper ones only UIII, and the middle ones unresolved mixtures of different compositions. The exact distribution of the isomers along the zone varies largely with the size and composition of the original sample. When a pilot column has been processed with a mixture of about 0.5 mg, the whole fluorescent zone may be divided into seven or even five fractions, with the top and bottom fractions thicker than several cm and the middle ones thinner than 0.5 cm. Oftentimes only about 0.5 cm of the zone somewhere below the middle line was found to contain both isomers. It is advisable to remove the fractions from both ends of the column and to use a separate filter tube for the elution of each fraction in order to avoid any unnecessary contamination.

A larger column of 3×60 cm was used for samples up to 2 to 3 mg and was packed with Hyflo Super-Cel to about three-fourths full. When the sample was properly introduced, it was developed first by 150 ml of benzene and then 150 ml portions of chloroform-benzene (1:2) mixture containing, respectively, 0, 3, 6, and 9 drops of ethyl alcohol. The development took 2 to 3 hours. The rest of the procedure was the same as before. Here again the unseparated mixture of the isomers occupied about 1 to 2 cm of the total 20 to 25 cm zone.

Paper Chromatography—For the identification of uroporphyrins and their decarboxylation products, coproporphyrins, the paper chromatographic methods of Falk and Benson (12) and of Chu, Green, and Chu (13) were, respectively, employed with some modifications as follows.

Chu-Green-Chu Method—The original chromatographic set-up was not changed. The first development was made in a mixture of 3 ml of kerosene and 3 ml of chloroform under a kerosene atmosphere for 20 minutes. The second development was made in 5 ml of kerosene and 0.9 ml of *n*-propyl alcohol, also under a kerosene atmosphere for an hour. The solvent front of the second dip was marked for the calculation of the R_F values. The

base line for spotting the samples was set at 1.2 cm from the lower edge of the paper, and the sizes of the sample and the spot were limited to about 0.5 γ and 2 mm in diameter, respectively.

Falk-Benson Method—The above chromatographic set-up was used. The first kerosene-chloroform development was also the same as above. The second development was made in a mixture of 4.4 ml of kerosene and 1.8 ml of dioxan (reagent grade) under a mixed atmosphere of the same amount of the developer placed in the outer beaker for 1 hour at 22–24°. At room temperature around 20° or 26°, the volume of kerosene was changed to 4.3 or 4.5 ml, respectively. The size of the sample was limited to about 0.3 γ , whereas the base line and the spot size were kept the same as in the Chu-Green-Chu method. With these modifications a better separation of the isomeric uroporphyrin esters and reproducible R_F values was obtained. The best result was achieved when the sample spot contained 50 per cent of a mixture of equal amounts of the two pure isomers. By addition of such a carrier, the minor constituent of the original sample was increased to a value greater than 25 per cent of the total, as observed on the paper chromatogram, and consequently the relative intensity of each of the two fluorescent spots would be 25 to 75 per cent of the total, which was found to be the most satisfactory range for estimation of isomeric proportion.

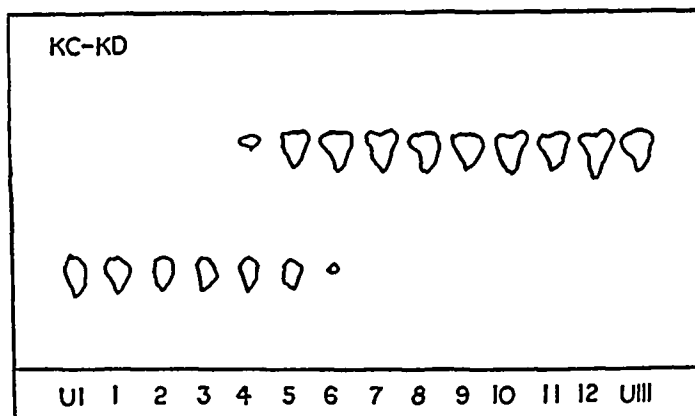
Results

Separation of Uroporphyrins I and III—The crude esters of uroporphyrins isolated from urine of Patients 2, 3, 4, and 6 were all obtained in hair-like crystals, but with wide melting point ranges between 265–280°. Each sample was purified by the use of a second column before it was introduced into a long Hyflo column as described under chromatography. It was a continuous chromatogram with a higher concentration at the lower end (Text-fig 1, B). The eluate of each fraction was analyzed by the modified Falk-Benson method. The paper chromatogram of a typical pilot batch was shown in Text-fig 2. Melting points were determined after crystallization from chloroform-methanol. The products, melting below 260° and showing the same R_F values as Rimington's UIII, were mixed as the crude UIII. Those melting above 290° and having the same R_F value as Watson's UI were mixed as the crude UI, and then further purified on long Hyflo columns to eliminate any possible contamination with one another. The pure UI ester thus obtained melted at 293–295°, and the pure UIII ester melted at 258–261° (Fig 1, a and b). Both showed the same absorption maxima at 502, 535, 571, and 626 m μ in chloroform.

In order to determine the efficiency of the Hyflo column in the separation of the isomers, an artificial mixture of 245 γ of Watson's UI ester and

196 γ of Rimington's UIII ester was subjected to the same procedure. As a result, 145 γ of UI (m p 293°), 118 γ of UIII (m p 262°), and three small fractions of unresolved isomers totaling 51 γ were obtained. As shown by paper chromatography, these fractions contained about 32 γ of UI ester and 19 γ of UIII ester. Regardless of the loss on the column, an over-all yield of 56.4 per cent of UI and 43.6 per cent of UIII against 55.5 per cent of the former and 44.5 per cent of the latter in the original sample was accounted for.

The experiments on decarboxylation were carried out in sealed tubes according to the procedure of Edmondson and Schwartz (14). The esteri-



TEXT-FIG. 2. Paper chromatogram of esters of UI and UIII separated on a typical pilot Hyflo column. UI and UIII stand for uroporphyrin I and III markers, Nos 1 to 12, for eluates from respective sections of the column (see Text-fig. 1, B). The solvent mixture KC-KD consisted of 3.3 ml of kerosene-chloroform, followed by 4.418 ml of kerosene-dioxan at 22°.

fied decarboxylation product of each isomer revealed a single zone on a Hyflo column and a clear spot corresponding to methyl ester of coproporphyrin I or III on a paper chromatogram, according to the modified Chu-Green-Chu method. They were readily crystallized from chloroform-methanol in the well known crystalline forms of coproporphyrin I (copro-I) ester, m p 248–250°, and copro-III ester, m p 146–148° and 165–170° (Fig. 1, c and d). Their absorption maxima in chloroform were 499, 533, 568, and 622 $m\mu$. Other data are listed in Table I.

Waldenström Porphyrin.—Although the patients under investigation suffered porphyria of different types, the crude Waldenström porphyrin ester prepared from Patients 2, 4, 5, and 6 crystallized in a more or less similar form with a melting point of about 255–265°. Their chemical compositions were, however, different, as shown by the paper chromatography (Text-fig. 3). Patients 2 and 6, both patients with cutanea tarda,

and Patient 5, with a mixed type, showed, besides uroporphyrins, the presence of more than 10 per cent of a heptacarboxylic porphyrin of the III series, "7III," whereas Patient 4, with an acute type, discharged almost exclusively uroporphyrins. The Waldenstrom porphyrin first isolated by Waldenstrom *et al* (1, 2) was also from a patient with acute porphyria. In each case the total amount of porphyrins extracted with

TABLE I
Major Porphyrins Isolated from Porphyria Patients

Patient No	Urine, ml	Uroporphyrin I				Uroporphyrin III				"7III" porphyrin	
		Weight	M p	Per cent composition determined by		Weight	M p	Per cent composition determined by		Weight	M p
				Paper chromatography	Hyflo column			Paper chromatography	Hyflo column		
		mg	°C			mg	°C			mg	°C
2	19,600	49.5	295	77	78	14.6	260	23	22	19.5	218
3	8,280	14.4	293	65	62	8.8	261	35	38	13.2	218
4	88	0.335	294	50	53	0.295	260	50	47	0.002	
5	95	0.030		67		0.015		33		0.008	
6	60	0.560	293	59	60	0.370	261	41	40	0.168	218

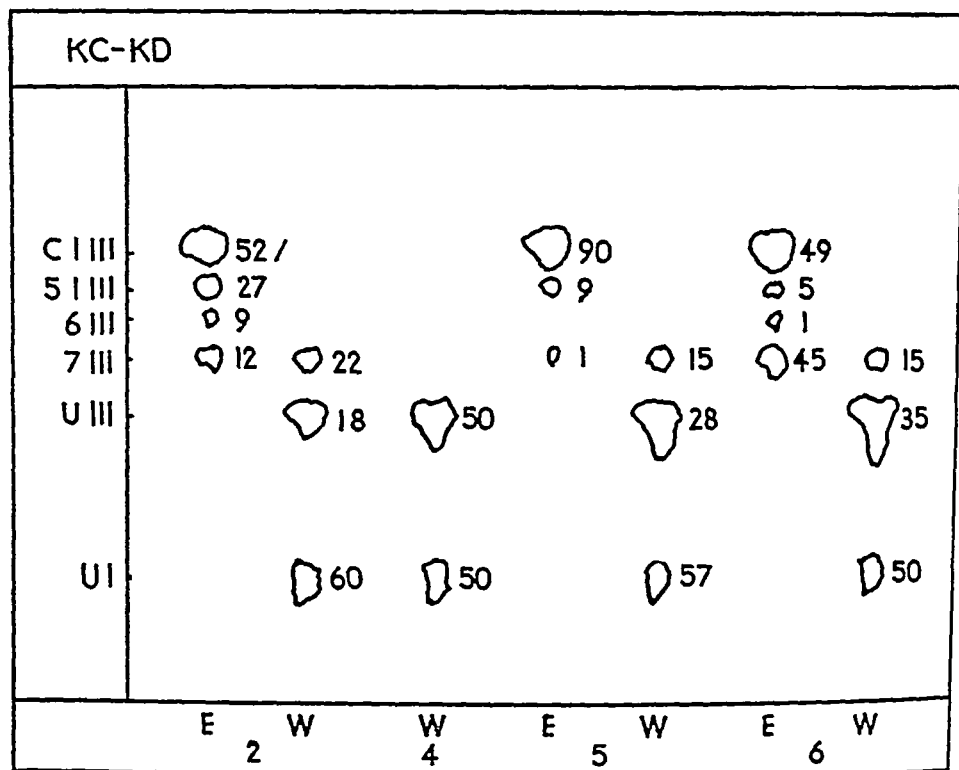
Decarboxylation products

	U-I → copro I			U III → copro III			"7III" → copro III		
	Weight of U I	Weight of copro I	M p of copro I	Weight of U III	Weight of copro III	M p of copro III	Weight of "7III"	Weight of copro III	M p of copro III
	γ	γ	°C	γ	γ	°C	γ	γ	°C
2	220	140	252	285	195	149/170	210	135	150/173
4	120	53	250						
6	125	60	251	75	32	148/162	60	28	146/170

ether and then ethyl acetate was the same as that by the adsorption on talc. With the urine from Patient 4 no extraction with ether was performed, and the minor constituents, "7III," "6III," etc., amounting to much less than 0.5 per cent, were also found in the ethyl acetate extract on the Hyflo column (Text-fig 1, D).

Of those minor constituents isolated (to be reported later), the "7III" porphyrin is of special interest. Apparently its methyl ester is similar to the product "208" reported by Grinstein *et al* (4) and the "pseudouro," m p 211–216°, reported by Falk *et al* (15). It was readily separated

from other members on a primary Hyflo column and was found to be present in urine samples of all the patients and also in liver and serum of Patient 7. After chromatographic purification, it crystallized (not as readily as Waldenström ester) from chloroform and methanol as fine wool-like needles, m p 217–218° (Fig 1, e). Its absorption in chloroform at



TEXT-FIG 3 Paper chromatogram of esters of urinary porphyrins from different patients (Nos 2, 4, 5, and 6), with their intensity distribution expressed in percentage. *E* stands for ether-extracted portion, *W* for ethyl acetate extract or Waldenström porphyrin.

502, 537, 571, and 625 $m\mu$ was observed. The free porphyrin showed its best approximate relation to a heptacarboxylic porphyrin on the lutidine-water paper chromatogram (16). The HCl number of the porphyrin is 0.03 and that of its ester is 0.2, as compared with 0.08 and 0.35 for coproporphyrin and its ester, respectively. The presence of this and other porphyrins in the ether extract (Text-fig 3) might have caused some variations in the estimation of coproporphyrins of certain crude preparations. By decarboxylation, the "7III" porphyrin yielded 90 per cent of coproporphyrin III, its ester melting at 148–150° and 169–173° (Fig 1, f).

For the separation of the Waldenstrom porphyrin ester, both the MgO^3 column, according to Nicholas (6), and the Hyflo column were used for comparison. The difference of the two chromatograms with the product from Patient 4, m p 256–265°, was shown in Text-fig 1, *C* and *D*. The Hyflo column gave a clear separation of the “7III” and others. Similar results were obtained from another Waldenstrom porphyrin from Patient 6, m p 251–262°, on the MgO and Hyflo columns. Although it was known from paper chromatography (Text-fig 3) that Patient 6 contained about 15 per cent of the “7III” porphyrin, yet the MgO column still showed no separation of this porphyrin and uroporphyrins. However, when the solvent system, benzene-methanol, was changed to a proportion of 100:2 instead of 100:4, as originally specified (6), the “7III” ester and uroporphyrin esters were also separated on an MgO column (Text-fig 1, *E*).

The uroporphyrin isomers, separated from the Waldenstrom porphyrin by a primary Hyflo column, were then separated on a long Hyflo column as described before. The isomeric composition of UI and UIII found by the Hyflo column agreed with that found by the paper chromatography (Text-fig 3). The results are listed in Table I.

A small sample of a considerably purified⁴ typical Waldenstrom porphyrin ester was kindly sent to us by Dr Gray. It melted, according to Dr Gray, at 251–256°. From our observation through a microscope, it melted mainly as reported but not completely until 264–266°, even with a single crystal. Its absorption maxima in chloroform were found at 502, 536, 570, and 626 $m\mu$. On a paper chromatogram it revealed itself as a mixture of uroporphyrins with UIII predominating and without any detectable amount of the “7III” porphyrin. The small sample, 195 γ , was chromatographed on a special 0.75 \times 40 cm Hyflo column, with heavier suction throughout the development. Due to the high vacuum created in the system, the eluate froze at the outlet of the column. Despite a warm air current occasionally blowing around the exit to ease the passage of solution, the porphyrin front reached only a little over one-half of the column after 5 hours. However, 10 γ of UI ester, m p 290°, and 78 γ of UIII ester, m p 254–260° (Fig 1, *g* and *h*), were isolated from this column. One-third of the sample was still a mixture. By decarboxylation, the UIII yielded 41 γ of coproporphyrin III ester.

DISCUSSION

Hyflo Super-Cel has been found to be a better adsorbent for the separation of porphyrin esters than other materials tested, although the adsorb-

³ A sample of MgO with an iodine number of 29 was kindly supplied by Dr Charles Rosenblum, Merck and Company, Inc., Rahway, New Jersey.

⁴ Personal communications.

ing efficiency varies slightly from batch to batch. In order to remove any undesirable impurity, it should be washed with chloroform and dried thoroughly before use. The addition of a small amount of ethyl alcohol to the developing solvent helps the spreading of the uroporphyrin zone on the column, but larger amounts will prevent the spreading. Chloroform from different companies contains different amounts of alcohol, and that of the reagent grade is preferred.

Attempts have been made to separate esters of UI and UIII into distinct zones by increasing the length of the Hyflo column without much success. Besides the experimental difficulties encountered in the chromatography of Gray's product, a longer developing time (overnight) increases the possibility of partial hydrolysis of porphyrin esters, a phenomenon which has also been noticed by other investigators (17) working with alumina columns.

Falk and Benson's paper chromatographic method (12) for uroporphyrin esters has been modified. By changing the proportion of solvents with change in room temperature and limiting the size of the sample and the developing time, a better chromatogram with reproducible R_f values was obtained. As mentioned before, the satisfactory range for estimation of isomeric proportion by this method is limited. Therefore, when this method is used as a guide for the column chromatography of uroporphyrins, it is advisable to treat those segments nearest to the unresolved ones (e.g. Nos. 3 and 7 in Text-fig. 2) from a long Hyflo column still as crude products in spite of their satisfactory melting points.

The esters of UI and UIII separated by the Hyflo column were respectively identified with Watson's UI and Rimington's UIII ester and, also, their decarboxylation products were identified. The possible existence of uroporphyrins II and IV in nature, as was pointed out by MacDonald (18), would add some doubt as to the purity of all the uroporphyrins thus far isolated from natural substances, although these contaminants seem to be small.

The identity of our product, the "7III" porphyrin, with "208" of Watson *et al.* (5) is yet to be established because there are some small differences, such as in melting point, HCl number, and sources of materials. Watson *et al.* prepared their "208" from pooled urine of a group of patients with acute porphyria, whereas our "7III" was mainly from patients with cutanea tarda. We did, however, isolate a small amount of "7III" porphyrin from Patient 4, also with an acute case of porphyria. Furthermore, we have found a heptacarboxylic porphyrin of series I ("7I") on decarboxylating UI, its methyl ester melting around 240°. The "7I" porphyrin yielded copro-I on further decarboxylation.

From our findings thus far, we are inclined to believe that the so called

Waldenstrom porphyrin is a group of ethyl acetate-extractable porphyrins. From urine of patients with porphyria, it is a mixture of UI, UIII, and "7III," and possibly some "7I" porphyrins. The exact percentage composition of such a mixture varies with the patients and, to a lesser degree, with experimental conditions. As Diesel *et al* (19, 20) have pointed out, even pure UI can be extracted quantitatively into ethyl acetate in a strongly buffered solution. In some instances the percentage of "7III" porphyrin may be so small that the product will be the same as the "type B Waldenstrom porphyrin" of Watson *et al* (4, 5, 9, 10). This, of course, does not exclude the possibility of a molecular compound of UI and UIII with certain definite composition, but experimental evidences, including the x-ray diffraction patterns (8, 10), indicate that the chance is small. Whether the presence of the "7III" porphyrin has some special relation to patients with cutanea tarda remains to be seen.

SUMMARY

1 The methyl esters of uroporphyrins I and III (UI and UIII) isolated from urine and liver of patients with porphyria, including intermittent acute, cutanea tarda, and mixed types, have been separated on a Hyflo Super-Cel column by using chloroform-benzene as the developing solvent, thus confirming the presence of disputed uroporphyrin III in urine from patients with porphyria.

2 The procedure of conducting Hyflo columns for the efficient separation of porphyrin esters as well as the uroporphyrin isomers has been described in detail.

3 A less familiar heptacarboxylic porphyrin of series III has been isolated from these patients. It is readily separable from uroporphyrin esters on a Hyflo or modified MgO column. The possible identity of this porphyrin with Watson's "208" porphyrin is discussed.

4 The Waldenstrom porphyrin has been prepared from urine of patients with acute porphyria as well as with cutanea tarda. Chromatographic analyses have revealed its nature as a mixture of UI, UIII, and "7III" porphyrins of varying proportions from patient to patient. The possibility of a molecular compound with certain definite composition is mentioned.

5 The paper chromatographic methods of Chu-Green-Chu and Falk-Benson have been modified for the respective identification of coproporphyrins I and III, and uroporphyrins I and III.

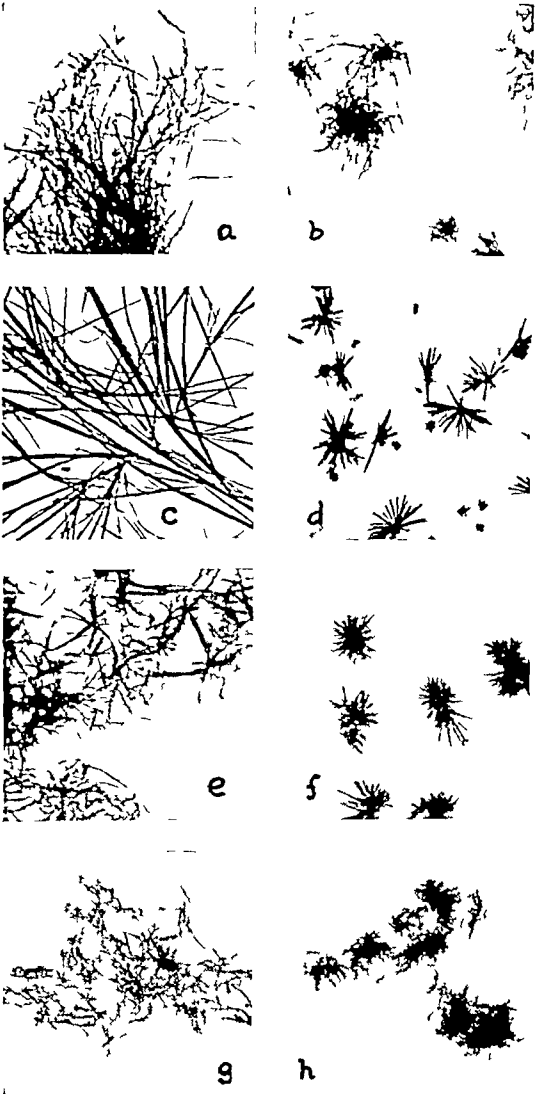
The authors wish to express their appreciation to Sister Agnes Ann Green for her interest in this investigation.

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EXPLANATION OF PLATE 1

Fig 1 (a) Crystals of uroporphyrin I methyl ester, m p 295°, (b) crystals of uroporphyrin III methyl ester, m p 261°, (c) crystals of coproporphyrin I methyl ester, m p 250°, obtained by decarboxylation of (a), (d) crystals of copro-III methyl ester, m p 148° and 170°, obtained by decarboxylation of (b), (e) crystals of "7III" porphyrin methyl ester, m p 218°, (f) crystals of copro-III methyl ester, m p 150° and 173°, obtained by decarboxylation of (c), (g) and (h) crystals of methyl esters of uroporphyrins I and III, respectively, separated from Gray's Waldenstrom porphyrin Magnification (e) $\times 145$, others $\times 70$



(Chu and Chu Uroporphyrins)

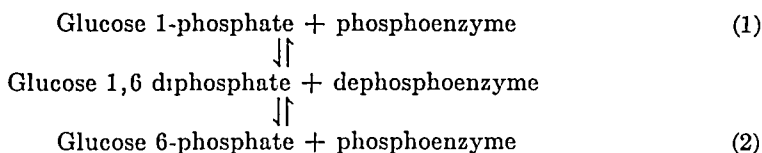
FURTHER STUDIES ON THE MECHANISM OF PHOSPHOGLUCOMUTASE, THE PHOSPHOENZYME BOND*

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The mechanism by which the enzyme phosphoglucomutase converts glucose 1-phosphate to glucose 6-phosphate was reported earlier (1, 2) and involves two distinct steps as follows



The validity of this mechanism was established by characterization and measurement of the component parts of the reaction (2). Thus the enzyme exists in two forms, a phosphorylated and a non-phosphorylated form. The phosphorylated mutase loses its phosphate by reacting with glucose 1-phosphate or glucose 6-phosphate to produce the diphosphate in each case. The diphosphate may then react with the dephosphorylated mutase. In such a reaction the C1-phosphate of the diphosphate may be transferred to the enzyme to produce the phosphorylated enzyme and glucose 6-phosphate. Alternatively, the C6-phosphate may be transferred and result in the formation of phosphoenzyme and glucose 1-phosphate.

The results reported in this paper indicate that the enzyme possesses 1 mole of phosphate per mole of enzyme and that this phosphate group is transferable to either of the two glucose monophosphates. The value for the standard free energy of hydrolysis of the enzyme-phosphate bond is found to be -3914 calories, based on a value of -3000 calories for the ΔF° of hydrolysis of the phosphate bond in glucose 6-phosphate (3). The standard free energy change of each step was calculated from the respective equilibrium constants k_1 and k_2 for Reactions 1 and 2 obtained at 30° at pH 7.5. $k_1 = 4.58$ and $k_2 = 3.76$, based on an over-all equilibrium constant, $K = 17.2$, for the whole reaction (4, 5). A preliminary account of this work was reported earlier (6).

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EXPERIMENTAL

The reaction was started (a) with glucose 1-phosphate and phosphoenzyme, (b) with glucose 6-phosphate and phosphoenzyme, or (c) with glucose 1,6-diphosphate and dephosphoenzyme. It was carried out under the following standard conditions: Mg^{++} , 1×10^{-3} M, histidine, 5×10^{-2} M, temperature, 30° , pH 7.4. The reaction was terminated by boiling in a water bath.

The glucose 1-phosphate and glucose 6-phosphate (Schwarz Laboratories, Inc.) used contained no measurable amounts of glucose 1,6-diphosphate. Glucose 1,6-diphosphate was obtained in part from the Schwarz Laboratories and in part through the kindness of Dr. Luis Leloir. Neither contained any measurable quantity of the monophosphates.

Preparation of Enzyme—Phosphoglucumutase was isolated in the crystalline form from rabbit muscle (4). It was particularly fortunate that virtually all the enzyme so isolated appeared as *phosphoenzyme*, as no detectable transfer of phosphate occurred when it was allowed to react at standard conditions with glucose 1,6-diphosphate (2).

The *dephosphoenzyme* was prepared by treating the phosphorylated enzyme with 4 to 20 times its concentration of glucose 1-phosphate, followed by 24 hours dialysis against tris(hydroxymethyl)aminomethane (Tris) buffer, 5×10^{-2} M, pH 7.4. This treatment was repeated three times. The enzyme was finally dialyzed for 48 hours against the same buffer. At this point it was fully active, yet was incapable of forming glucose 1,6-diphosphate in detectable quantities when allowed to react under standard conditions with glucose 1-phosphate. This is indicative of the loss of all transferable phosphate from the enzyme.

The enzyme was assayed by activity measurements under standard conditions in the presence of glucose 1,6-diphosphate as activator at a concentration of 5×10^{-6} M (2, 4). The calculated molar concentration of the enzyme was based on a molecular weight of 74,000 (7). Glucose 6-phosphate was assayed spectrophotometrically with the glucose-6-phosphate dehydrogenase system (8). Glucose 1,6-diphosphate was measured by its coenzymatic activity (9) or by measuring the glucose 6-phosphate upon hydrolysis in 0.1 N HCl for 10 minutes at 100° . Phosphorus was measured as the reduced phosphomolybdate (10) at $660 m\mu$ in the Beckman spectrophotometer with 10 per cent Elon (Eastman Kodak Company) as reducer, and total phosphorus determinations in enzyme samples were made as usual (11). For studies of the rate of the mutase reaction, glucose 1-phosphate was measured by the hydrolyzable phosphate liberated by 1 N H_2SO_4 in 3 minutes at 100° . In the quantitative study at equilibrium states of the phosphoglucumutase reaction, glucose 1-phosphate concentration was too low to measure accurately.

When either of the glucose monophosphates was allowed to react with the phosphoenzyme, the dephosphoenzyme formed was assumed to be equivalent to the glucose 1,6-diphosphate formed. The value for the phosphoenzyme at equilibrium was then equivalent to the phosphoenzyme added minus the dephosphoenzyme formed. When the reaction was started with glucose 1,6-diphosphate and dephosphoenzyme, the phosphoenzyme formed was taken to be equal to the sum of the glucose monophosphate values. The dephosphoenzyme concentration at equilibrium was calculated from the amount added less the phosphoenzyme formed. No account was taken of other possible equilibria involving Mg^{++} complex formation or non-specific adsorption of the enzyme protein.

Results

As indicated above, the enzyme exists in a phosphorylated and a non-phosphorylated form. However, crystalline phosphoglucumutase is wholly of the phosphorylated type (2). It is possible that the non-phosphorylated form cannot withstand the procedure used in the isolation of the enzyme (4) or that it does not crystallize under those conditions.

The evidence indicates that the phosphoenzyme has 1 mole of phosphorus per mole of protein. Assays of a number of preparations of dialyzed crystalline enzyme gave values varying between 0.7 and 1.3 moles of phosphorus per mole of enzyme. In one experiment, for example, when 0.202 μ mole of three times recrystallized phosphoglucumutase was dialyzed for 3 days with repeated changes of 0.02 M Tris buffer, pH 7.4, and assayed for total phosphorus, 0.185 μ mole of phosphorus was found. It was shown that all of this phosphorus could be transferred by enzymatic dephosphorylation. In such an experiment the phosphate group of the enzyme is completely transferred to the sugar phosphate to form the diphosphate (2). The transfer of phosphate from the enzyme is most active with glucose 1-phosphate and glucose 6-phosphate, but, judging by the activity of phosphoglucumutase with the monophosphate esters of mannose, galactose, and ribose, it is to be expected that the phosphate from the enzyme would transfer to these esters also to form the corresponding diphosphate and a dephosphorylated mutase, although at a much slower rate (12, 13). Glucose, fructose, galactose, mannose, and ribose do not react with the enzyme.

The quantitative data from which the equilibrium constants k_1 and k_2 were derived for the two steps in the mutase reaction are given in Table I. In order to obtain maximal accuracy in the quantitative determinations of the participants in the reaction, it was found necessary to use concentrations of the glucose phosphates falling within the same order of magnitude as those of the enzyme. This made it impossible to measure glucose 1-phosphate because of the unfavorable over-all equilibrium of the reaction

($K = 17.2$) It was found practical, therefore, to determine only the equilibrium constant of the second step (k_2) and derive that of the first step (k_1) from the over-all K and k_2 . The average value for k_2 was found to be 3.76

Since $K = k_1 \times k_2$, $k_1 = ((17.2/3.76)4.58)$, where $K = (\text{glucose 6-phosphate})/(\text{glucose 1-phosphate})$, $k_1 = ((\text{glucose 1,6-diphosphate})(\text{dephosphoenzyme})/(\text{glucose 1-phosphate})(\text{phosphoenzyme}))$, and $k_2 = ((\text{glucose$

TABLE I
Equilibrium Values for Step II of Phosphoglucumutase Reaction

Ex- peri- ment No	Glucose 1,6- diphosphate	Dephosphoenzyme	Glucose 6 phosphate	Phosphoenzyme	k_2
	μmole	μmole	μmole	μmole	
1	0.3200 (0.4250)	0.0060 (0.1060)	0.1000	0.1000	5.25
2	0.0720 (0.1225)	0.0090 (0.0570)	0.0480	0.0480	3.55
3	0.1760 (0.2260)	0.0085 (0.0585)	0.0980 (0.0480)	0.0500	3.28
4	0.0064	0.0064	0.0130 (0.0200)	0.0080 (0.0144)	2.73
5	0.0037	0.0037	0.0164 (0.0210)	0.0043 (0.0080)	5.16
6	0.0080	0.0080	0.0422 (0.0535)	0.0040 (0.0120)	2.64
7	0.0180	0.0180	0.0730	0.0200 (0.0380)	4.50
8	0.0490	0.0490	0.1420	0.0510 (0.0100)	3.00
Average					3.76

The reaction was carried out under standard conditions in 1 ml. of the reaction mixture as follows: histidine, 0.05 M, Mg^{++} , 1×10^{-3} M, pH 7.5, temperature, 30° , incubation time, 10 minutes. In Experiments 7 and 8 the reactants were glucose 1-phosphate, 0.095 and 0.2 μmole , respectively, as well as phosphoenzyme. The values in parentheses represent the initial concentrations of the reactants used.

6-phosphate)(phosphoenzyme) / (glucose 1,6-diphosphate)(dephosphoenzyme))

The standard free energy change of the respective steps of the reaction was calculated from the following equation (14)

$$\Delta F^\circ = -RT \ln K$$

$$\Delta F^\circ \text{ of Step I} = -914 \text{ calorie}$$

$$\Delta F^\circ \text{ of Step II} = -800 \text{ calorie at } 30^\circ$$

Total ΔF° of the over-all reaction based on K of 17.2 (4, 5) is -1714 calorie. Assuming that the ΔF° values for hydrolysis of the glucosyl phosphate bond of glucose 1-phosphate and glucose 1,6-diphosphate are

very nearly equal,¹ $\Delta F^\circ = -4714$ calorie, it follows that ΔF° for hydrolysis of the phosphoglucomutase-phosphate bond is equivalent to -3914 calorie, i.e. ΔF° for hydrolysis of the mutase $-P = \Delta F^\circ$ for hydrolysis of the glucosyl phosphate $-\Delta F^\circ$ Step II or $(-4714) - (-800) = -3914$ calorie

DISCUSSION

It appears that the phosphate bond in the enzyme is of the low energy type and at the hydroxyl phosphate ester level. It is unlikely that the bond would be of the enol, acyl, or guanidine phosphate type of high energy bond unless the protein moiety could so drastically alter the group potential of this type of bond as to bring it down to a low level. A pyrophosphate bond can readily be excluded because of the presence of only 1 mole of phosphate per mole of enzyme.

It is significant that the enzyme-phosphate bond is of the low energy type with a ΔF° for hydrolysis about midway between that of the glucosyl and the hydroxyl phosphate bonds. The standard free energy change of the whole system is therefore divided almost equally between the two steps of the reaction. This serves to lower the activation barrier for the reverse reaction and render the latter more efficient.

The demonstration of two definite steps in the phosphoglucomutase reaction as formulated above places this enzyme system well within the realm of ordinary chemical reactions. It does not necessitate a special type of complex comprising the enzyme and substrates with a template or "a lock and key fit" for surface catalysis (15, 16). This type was advanced by Michaelis (15) and is currently considered to include a so called active enzyme surface with anchoring points for the substrates. A Michaelis type complex is not needed to explain a state of substrate saturation as far as the mutase reaction is concerned. Such a state is obtained when one of the two steps becomes rate-limiting. This does not imply that a transition state complex such as that ordinarily assumed in organic chemical reactions may not occur. In this instance the mutase would have two such transition complexes instead of one Michaelis complex.

SUMMARY

The equilibria for the two steps in the phosphoglucomutase reaction have been determined. While the value for over-all K of the reaction is

¹ A basis for this assumption exists in that close agreement has been shown for the standard free energy of hydrolysis of the phosphate bond on the 6 position of fructose in both the fructose mono- and diphosphates, determined with purified alkaline phosphatase (3)

$$K_{\text{monophosphate}} = ((\text{fructose})(\text{phosphate})/(\text{fructose 6-phosphate})(\text{H}_2\text{O}))$$

$$K_{\text{diphosphate}} = ((\text{fructose 1-phosphate})(\text{phosphate})/(\text{fructose 1,6-diphosphate})(\text{H}_2\text{O}))$$

$$\Delta F^\circ_{\text{monophosphate}} = -3370 \text{ calorie}, \Delta F^\circ_{\text{diphosphate}} = -3675 \text{ calorie}$$

17.2, the equilibrium constants for the two steps are $k_1 = 4.58$ and $k_2 = 3.76$. The standard free energy changes are, respectively, -914 calorie for Step I and -800 calorie for Step II. The free energy for hydrolysis of the enzyme-phosphate bond is -3914 calorie.

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PURINE CATABOLISM IN MOLYBDENUM DEFICIENCY*

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A low protein diet (1, 2) removed about three-fourths of the xanthine oxidase from the entire rat (3), while a purified diet containing tungstate reduced the tissue xanthine oxidase to a level at which it could not be detected manometrically (4), neither of these dietary procedures had any effect on the uric acid or allantoin excretion by this species. Chicks fed a tungstate-containing diet excreted a mixture of xanthine, hypoxanthine, and uric acid in response to the marked depletion of tissue xanthine dehydrogenase and molybdenum (4). Either (a) small amounts of xanthine oxidase persisted in the tissues of tungstate-fed rats but escaped detection by the manometric procedure, or (b) uric acid was formed in rats by a pathway which was not molybdenum-dependent, *e.g.* by the oxidation of inosinic acid to xanthylic acid (5) and further oxidation to uric acid ribotide. Such a possibility was explored previously with rat livers in which the xanthine oxidase had been removed by feeding a low protein diet, but no alternative pathway which bypassed the Mo-containing xanthine oxidase could be found (6).

In the previous study (6) an alternative system might have escaped detection if it were also removed from the liver by a protein deficiency. This problem has therefore been reinvestigated with livers from which the xanthine oxidase was removed by tungstate feeding. Although no xanthine oxidase activity could be detected manometrically in the deficient livers, enough of the enzyme was retained to account for the formation of the normal daily output of uric acid and allantoin by the deficient rats. From a study of net oxygen consumption as well as uric acid and allantoin production from various nucleoside and nucleotide substrates, there were no indications that xanthine oxidase was bypassed in the formation of uric acid by the rat.

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EXPERIMENTAL

Methods

Weanling or adult male albino rats were depleted of xanthine oxidase by feeding a purified casein diet (7) containing 72 mg of Na_2WO_4 per kilo for 2 weeks or longer. Control rats received the same diet except that it also contained 50 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ per kilo.

Liver xanthine oxidase activity was measured manometrically in the presence of methylene blue (8) with xanthine or hypoxanthine as substrate. For oxidation measurements of the other substrates, the main body of the Warburg vessels contained 1.55 ml of a 1:5:5 liver homogenate (whole or dialyzed for 18 hours against 10^{-4} M cysteine in 0.04 M phosphate buffer, pH 7.4), 0.15 ml of 0.05 M DPN¹ or water, and 0.15 ml of water or other agents as specified. The side arm contained 0.15 ml of 0.05 M substrate. In some experiments the DPN and substrate were both contained in the side arm and tipped in together. The center well contained 0.2 ml of 10 per cent KOH. After 40 minutes of incubation the contents of the side arm were mixed with those of the flask, and manometric readings were made every 20 minutes for 80 minutes. Aliquots were then deproteinized with a final concentration of 5 per cent trichloroacetic acid for measurements of uric acid (9, 10) and allantoin (11). Combined uric acid, such as the riboside, had to be hydrolyzed in order to obtain the Folin color, for this purpose HCl was added to aliquots of the trichloroacetic acid filtrate to give a concentration of 3.3 N, and the solutions were heated in a boiling water bath for 1 hour.

Results

The various substrates were oxidized by control liver homogenates from rats receiving both tungstate and molybdate in the diet at the following rates: xanthine,² 26, xanthosine, 48, hypoxanthine, 32, inosine, 48, IMP, 48, guanine, 31, guanosine, 52, GMP, 41, adenosine, 42, and AMP, 42 c mm of O_2 per 20 minutes. With xanthine oxidase-deficient livers from tungstate-fed rats, the homogenates did not oxidize any of the substrates. The addition of purified milk xanthine oxidase (12) to these deficient liver

¹ The following abbreviations are used: DPN, diphosphopyridine nucleotide, Mo, molybdenum, W, tungsten, IMP, inosine 5-phosphate, GMP, guanosine 5-phosphate, AMP, adenosine 5-phosphate, pteridylaldehyde, 2-amino-4-hydroxy-6-pteridinecarboxaldehyde.

² The xanthine, hypoxanthine, and guanine were obtained from the Schwarz Laboratories, Inc., guanosine, adenosine, and GMP from the Pabst Laboratories, inosine and xanthosine from the California Foundation for Biochemical Research, IMP and DPN from the Sigma Chemical Company, AMP from the Nutritional Biochemicals Corporation, and pteridylaldehyde from the Lederle Laboratories Division, American Cyanamid Company.

homogenates restored the oxidizing capacities to control levels. These results were essentially the same as those found with rats fed a protein-deficient diet (6) and showed that all of the substrates yielded hypoxanthine or xanthine in the course of their degradation and that the oxygen consumed was due to the action of xanthine oxidase on these two substrates.

Since DPN could participate in a possible alternative nucleotide or nucleoside oxidase system, such as the inosinic acid dehydrogenase of rabbit bone marrow (5), and because of the instability of DPN in homogenates (13), the rat liver homogenates in another series of experiments were fortified with DPN. Endogenous substrates were first removed by dialysis. Methylene blue was also added as an additional electron carrier to oxygen. The results with control livers from rats fed W-Mo were essentially the same as those when DPN was omitted: xanthine, 33, xanthosine, 46, hypoxanthine, 41, inosine, 49, IMP, 49, guanine, 35, guanosine, 55, GMP, 48, adenosine, 50, and AMP, 55 c mm of O_2 per 20 minutes. Again, none of the substrates was perceptibly oxidized under these conditions by deficient livers from W-fed rats, no evidence could be obtained that xanthine oxidase was being bypassed through a direct oxidation of the nucleotides or nucleosides.

Small amounts of xanthine oxidase were present in Mo-deficient rat tissues, but escaped detection by the manometric procedure. The presence of the enzyme was established by measuring the allantoin produced by the crude homogenates. Allantoin was determined after incubating the homogenates with or without substrates as previously described. The following homogenates of deficient livers from W-fed rats were used: (a) whole homogenate with substrate tipped in at 40 minutes, (b) substrate and DPN tipped in together, (c) whole homogenate containing an amount of purified milk xanthine oxidase which assayed 44 c mm of O_2 per 20 minutes with hypoxanthine, and (d) dialyzed homogenate. Both whole and dialyzed liver homogenates from control rats fed the W-Mo diet were also studied. By manometric assay the deficient livers showed no xanthine oxidase activity, while the control liver without dialysis had an activity of 15 c mm of O_2 per 20 minutes, and another liver after dialysis had an activity of 24.

As shown in Table I, the undialyzed and dialyzed deficient homogenates without added substrate contained 20 γ and 4 γ of allantoin per vessel, respectively, before incubation (zero time blank). After incubating for 120 minutes, each vessel contained 190 γ . The addition of 38 γ of 6-pteridylaldehyde, a xanthine oxidase inhibitor (14), allowed the formation of only 45 γ of allantoin. More hypoxanthine and xanthine were formed in the homogenate (presumably from nucleic acids) during the incubation period than could be oxidized by the small amount of xanthine oxidase present because (1) added uric acid formed about 1000 γ of allantoin, and

(2) the addition of milk xanthine oxidase increased the allantoin formation to 536 γ . Since this occurred in the blank Warburg vessel as well as in the one containing added substrate, no difference in oxygen consumption between the two flasks could be observed, hence, no xanthine oxidase activity could be observed manometrically, and small amounts of xanthine oxidase escaped detection by this procedure.

TABLE I
Allantoin Formation in Rat Liver Homogenates

Substrate	γ allantoin formed per 283 mg fresh liver per 120 min					
	Deficient liver homogenate				Control liver homogenate	
	Whole	Whole + DPN	Whole + milk xanthine oxidase	Dialyzed	Whole	Dialyzed
None (zero time blank)	20	26	40	4	48	18
"	190	159	536	190	404	371
Pteridylaldehyde (38 γ)			263	45	53	33
Xanthine*	120	137	1590	140	786	1100
" + pteridylaldehyde			570	45	53	56
AMP	110	130	1690		828	1340
GMP	170	148	1190		870	1090
IMP	120	133	704		1012	985
Xanthosine	130	152	1540		1355	
Inosine	120	107	1800		870	1120
Adenosine	100	111	1190		603	1075
Guanosine	170	163			1072	1490
Uric acid riboside	850					
Allantoin	1480	1366	1790		1072	1240
Uric acid	1000	985	1520			1090

* 0.15 ml of 0.04 M xanthine, uric acid, or uric acid riboside and 0.15 ml of 0.05 M solutions of the other substrates were used.

The small amount of xanthine oxidase present in the tissues of Mo-deficient rats was adequate for the production of the amount of uric acid and allantoin normally excreted by the rat. The formation of 0.19 mg of allantoin in 2 hours by 0.28 gm of liver was sufficient to account for the excretion of 12 to 15 mg of allantoin per 100 gm rat per 24 hours.

None of the substrates tested produced more allantoin than was formed from the deficient homogenate itself, unless xanthine oxidase was also added (Table I). Since HCl hydrolysis of the trichloroacetic acid filtrates produced no additional uric acid, and since uric acid riboside, prepared from beef blood (15), was converted by the homogenates to allantoin as

shown in Table I, there was no evidence for the formation of uric acid ribotide or riboside from the nucleotide or nucleoside substrates

DPN Catabolism by Rat Liver

Enzymes which cleave DPN form adenylic acid (13, 16), and the latter is readily converted to hypoxanthine by a liver homogenate (6). The addition of DPN alone to a rat liver homogenate increased the oxygen uptake and allantoin production (Table I) more in those livers containing xanthine oxidase than in those deficient in this enzyme. To test the possibility that a large portion of the increased oxygen consumption from added DPN was due to the oxidation of the derived hypoxanthine, DPN oxidation was measured manometrically in a Mo-deficient rat liver homogenate fortified with milk xanthine oxidase, 0.15 ml of 0.05 M DPN was tipped in from the side arm. Table II shows the effect of xanthine oxidase

TABLE II
*Effect of Milk Xanthine Oxidase on Oxidation of DPN
in Mo-Deficient Rat Liver Homogenate*

	C mm O ₂ per hr
Dialyzed homogenate alone	40
“ “ + xanthine oxidase	66
“ “ + DPN	78
“ “ + “ + xanthine oxidase	265
“ “ + hypoxanthine + xanthine oxidase	270

on the oxygen uptake in the presence of added DPN. With xanthine oxidase present, the net oxygen consumption with DPN as substrate was close to that found with hypoxanthine, DPN was therefore converted to hypoxanthine by the liver homogenate as rapidly or more rapidly than the latter could be oxidized (3.5 mg or more of DPN were decomposed per hour by 283 mg of fresh homogenized liver). When 5 mg of DPN were incubated with a dialyzed control rat liver homogenate for 140 minutes, 1535 γ of allantoin were formed, while a similar control flask without added DPN yielded 426 γ (a net production of 1109 γ of allantoin from the DPN, theoretical = 1185 γ). The presence of 15 mg of nicotinamide (6×10^{-2} M solution as suggested by Zatman *et al* (17)) reduced the net allantoin formed to 808 γ , this confirmed some stabilizing effect of the nicotinamide on DPN in the crude liver homogenate.

Pigeon Liver Homogenates

Pigeon liver was tested for its ability to oxidize purine ribosides or ribotides because it does not contain any xanthine oxidase, any oxygen con-

sumption from added nucleotides or nucleosides must be due to the oxidation of the combined purine or due to the metabolism of the ribose portion

In the absence of added DPN, the dialyzed homogenates of normal pigeon liver oxidized the nucleoside substrates only slightly (1 to 3 c mm of O_2 per 20 minutes) and did not oxidize the free purines or the nucleotides. With 5 mg of DPN added (with or without methylene blue), the nucleosides gave a net increase in the rate of oxygen consumption (guanosine 26, xanthosine 17, adenosine 18, and inosine 32 c mm of O_2 per 20 minutes), but yielded a net total consumption of only 50 to 70 c mm of O_2 . The rate of oxygen consumption with the nucleotide substrates was much less, even in the presence of 0.001 M $MgSO_4$. Free purines gave no measurable increase in oxygen uptake.

If this amount of oxygen were used for oxidizing the purine component to uric acid, from 700 to 1000 γ of uric acid should have been produced from guanosine and xanthosine or half this amount from adenosine and inosine. Actually the hydrolyzed trichloroacetic acid filtrates contained only small amounts of material which reduced the uric acid reagent, this was equivalent to no more than 25 γ of uric acid, and this amount was found also in the blank homogenates without added substrate. When an equivalent amount of uric acid riboside was incubated with the homogenate, about half of it was split to free uric acid, and the remainder was released upon acid hydrolysis. Therefore the purine derivatives were not oxidized to uric acid riboside by pigeon liver homogenates.

The increased oxygen consumption observed with the ribosides probably resulted from the release and metabolism of the ribose portion of the molecule. Free ribose was not oxidized, but the substitution of an equivalent amount of ribose 5-phosphate for the nucleosides produced a total net oxygen consumption of 72 c mm. This implies the formation of a phosphorylated ribose rather than a free ribose in the degradation of these purine ribosides by pigeon liver. The simultaneous formation of free purines was confirmed by the addition of purified milk xanthine oxidase to the pigeon liver homogenate and measurement of the amount of uric acid formed (Table III). In general, uric acid formation tended to parallel the oxygen consumption, this would be expected if the ribose were oxidized after being released from the purine.

Much more uric acid was formed from the nucleosides than from the nucleotides, hence, there was a greater stability of the nucleotides in pigeon livers. Such was not the case with rat liver (Table I). This difference was due to a lower 5'-nucleotide phosphatase activity in pigeon liver as compared with that in rat liver. The conditions for the phosphatase assays were the same as those used for the measurement of allantoin and uric acid formation (1.7 ml of 1.6 homogenate, pH 7.4, 0.15 ml of 0.05 M

substrate, 2 ml volume, incubated at 38° in air for 60 minutes) When the substrate was disodium phenyl phosphate, phenol liberation was measured by the method of King and Armstrong (18) Nucleotide phosphatase activity was measured by determining the increased inorganic phosphate in a 5 per cent trichloroacetic acid filtrate of the homogenate after incubation with nucleotides (19) The liver homogenates were dialyzed against water for 18 hours and then buffered to pH 7.4 with 0.04 M tris(hydroxymethyl)aminomethane, $MgSO_4$ was added to give a 0.001 M concentration

Under these conditions there was essentially no difference in the phos-

TABLE III
*Formation of Uric Acid from Various Substrates by Pigeon Liver
Homogenates Fortified with Milk Xanthine Oxidase*

Substrate (0.05 M)	γ uric acid formed per 283 gm. fresh liver per 100 min. *	
	Whole homogenate	Dialyzed homogenate
Xanthine (0.037 M)	752	604
Hypoxanthine	717	802
Guanine†	500	
DPN	25	
Xanthosine	355	123
Inosine	627	485
Adenosine	537	262
Guanosine	757	793
AMP	201	92
GMP	98	36
Inosinic acid	138	39
No substrate	140	57

* Total uric acid minus uric acid formed without added substrate

† Added as a suspension

phatase activities of rat and pigeon livers when phenyl phosphate was used as the substrate (whole rat liver homogenate, 17.7, dialyzed homogenate, 23, whole pigeon liver, 23, and dialyzed, 16.7 μmoles of phenol per hour per gm. of fresh liver) However, the rate of phosphate release from the nucleotides was appreciably lower in dialyzed pigeon liver than that in rat liver The phosphate released (micromoles per hour per gm. of fresh liver) by pigeon and rat livers, respectively, was as follows from AMP, 6.4 and 15.5, from GMP, 2.6 and 9.9, and from IMP, 2.8 and 9.9

These results are compatible with the interpretation that the oxygen which is consumed in the metabolism of nucleosides and nucleotides by pigeon liver homogenates is used in the metabolism of the ribose portion of the molecules The nucleotides are more stable in pigeon liver homoge-

nates than the nucleosides and are more stable in pigeon liver than in rat liver homogenates. The relatively poor 5'-nucleotide phosphatase activity in pigeon liver undoubtedly contributes to the usefulness of this tissue for the study of nucleotide synthesis.

SUMMARY

Homogenates of livers from rats depleted of Mo and xanthine oxidase by tungstate feeding did not show an increased oxygen uptake with free purines or their nucleosides or nucleotides as substrates. The addition of diphosphopyridine nucleotide (DPN) and methylene blue did not restore any of these oxidizing capacities, but milk xanthine oxidase did. Small amounts of xanthine oxidase were present in such livers, but escaped detection by the manometric procedure because endogenous substrates were being oxidized maximally in the blank vessel, and additional substrate could not increase this rate. The presence of xanthine oxidase was demonstrated by measuring allantoin formation from the various substrates, and enough xanthine oxidase was found to account for the formation of the uric acid and allantoin normally excreted by the rat. No evidence could be obtained that uric acid was formed in rat liver by any mechanism other than xanthine oxidase.

Normal pigeon liver is devoid of xanthine oxidase, but gave significant oxygen uptake with the purine nucleosides when DPN was also added. However, no uric acid was formed, and the increased oxygen consumption could be attributed to a metabolism of the ribose moiety. Nucleotides were more stable than nucleosides in pigeon than in rat liver homogenates, this difference was due to a lower 5'-nucleotide phosphatase activity in pigeon liver.

Much of the additional oxygen uptake resulting from the addition of DPN to a rat liver homogenate could be attributed to the oxidation of its purine component.

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ACETALDEHYDE OXIDATION IN MOLYBDENUM DEFICIENCY*

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Xanthine oxidase (1) and liver aldehyde oxidase (2) are molybdenum-containing enzymes, and both are capable of oxidizing aldehydes. Another aldehyde-oxidizing enzyme utilizing diphosphopyridine nucleotide (DPN) as a cofactor has been partially purified from liver (3), and the oxidation of acetaldehyde by crude liver extracts requires DPN (4). This study was designed to see whether the DPN enzyme was also Mo-dependent and which of these systems was primarily responsible for acetaldehyde oxidation in dialyzed liver homogenates. Rats were fed a diet containing tungstate in order to deplete the tissues of Mo-containing enzymes (5). The rate of acetaldehyde oxidation in liver homogenates was measured in the presence and absence of DPN. It was found that, while the capacity to oxidize acetaldehyde in the absence of DPN was virtually eliminated by the molybdenum deficiency, the oxidation of acetaldehyde in the presence of DPN was not affected. Feeding a protein-free diet caused the virtual elimination of all the aldehyde-oxidizing enzymes studied.

EXPERIMENTAL

Methods

3 to 6 week-old weanling albino rats were fed the following diets for at least 2 weeks: (1) Purina dog chow, (2) a purified 24 per cent Labco casein diet (6), (3) the purified diet containing 72 mg of Na_2WO_4 per kilo, (4) the purified diet containing 72 mg of Na_2WO_4 and 50 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ per kilo, and (5) a protein-free diet (6). In previous studies the purified tungstate-containing diet removed most of the xanthine oxidase and molybdenum from the liver (5). A protein-free diet also depleted the liver of xanthine oxidase and molybdenum (7) and produced some loss of a large variety of liver enzymes (8). In the present experiments, the xanthine oxidase level was used as an index of the effectiveness of the various diets in producing these changes.

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Concentrated liver homogenates (1:3) were dialyzed for 18 hours against water and then diluted with water and phosphate buffer, pH 7.4, to give a final 1:6 dilution of the liver in 0.04 M phosphate. Xanthine oxidase activities were measured manometrically in the presence of methylene blue (9). Acetaldehyde oxidation was measured by the anaerobic methylene blue decolorization time (10) as follows: 1 ml of the dialyzed 1:6 homogenate and sufficient 0.04 M phosphate buffer, pH 7.4, to give a final total volume of 2.5 ml were measured into each of four Thunberg tubes, all side arms contained 0.1 ml of 0.0113 M ($1.13 \mu\text{moles}$) of methylene blue. The side arm of Tube 1 also contained 0.1 ml (1 mg) of DPN and 0.1 ml of 1 M acetaldehyde. Tube 2 contained DPN without aldehyde and served as a blank, the difference in decolorization time between Tubes 1 and 2 was a measure of the combined activities of all the acetaldehyde-dehydrogenating enzymes (DPN-linked aldehyde dehydrogenase, aldehyde oxidase, and xanthine oxidase). The DPN was omitted from Tubes 3 and 4, acetaldehyde was added to the side arm of Tube 3, while Tube 4 served as a blank. With this pair was measured the action of the aldehyde dehydrogenases which were independent of DPN (aldehyde oxidase and xanthine oxidase).

All tubes were evacuated and flushed with nitrogen several times, after warming at 38° for 3 minutes, the contents were mixed, and the decolorization times were measured. The micromoles of methylene blue decolorized per minute were calculated for each tube, and the amount specifically decolorized by the DPN-linked enzyme was obtained by subtracting the values found in Tubes 2 and 3 from those in Tube 1. The decolorization time in Tube 4 was always greater than 60 minutes and was negligible. A linear relationship between the amount of normal dialyzed liver (0 to 1 ml) and the micromoles of methylene blue decolorized by acetaldehyde and DPN was found. The slope of this line, which passed through the origin, was equivalent to the reduction of $0.68 \mu\text{mole}$ of methylene blue per minute by 1 ml of the 1:6 homogenate.

Results

The DPN-linked aldehyde dehydrogenase was responsible for most of the activity of the dialyzed liver homogenate in the reduction of methylene blue (Table I). In normal livers the DPN-linked enzyme was responsible for the reduction of about $0.5 \mu\text{mole}$ of methylene blue per minute, and this activity was not Mo-dependent, it remained relatively constant as the xanthine oxidase and other Mo enzymes were removed by tungstate feeding. The aldehyde enzymes not requiring DPN normally reduced about $0.1 \mu\text{mole}$ of methylene blue per minute. This latter activity was Mo-dependent, since it tended to parallel the xanthine oxidase and to disappear with it as a result of tungstate feeding. The protein-free diet removed

about 90 per cent of the DPN-linked and all of the Mo-dependent aldehyde-oxidizing systems from the liver preparation. In previous experiments (7) it was found that rats which were fed a protein-free diet metabo-

TABLE I
Aldehyde Dehydrogenase Activities in Control and Mo-Deficient Rat Livers

Experiment No	Diet	No of rats	Additions to homogenate + methylene blue		Decolorization time	μmole methylene blue reduced per min		Xanthine oxidase
			Tube No				Due to DPN linked enzyme (Tube 1 - Tubes 2 + 3)	
1	Dog chow	13	1	Aldehyde + DPN	1 65	0 68	0 42	25
			2	DPN	6 91	0 16		
			3	Aldehyde	11 58	0 10		
			4		>60			
2	24% purified casein	5	1	Aldehyde + DPN	1 58	0 72	0 53	11
			2	DPN	10 26	0 11		
			3	Aldehyde	14 50	0 08		
			4		>60			
3	Purified casein + W	8	1	Aldehyde + DPN	1 87	0 60	0 56	0*
			2	DPN	32 20	0 04		
			3	Aldehyde	>60			
			4		>60			
4	Purified casein + W + Mo	5	1	Aldehyde + DPN	1 60	0 71	0 44	13
			2	DPN	6 06	0 19		
			3	Aldehyde	13 80	0 08		
			4		>60			
5	Protein-free	12	1	Aldehyde + DPN	16 0	0 07	0 05	4
			2	DPN	45 0	0 02		
			3	Aldehyde	>60			
			4		>60			

* The livers with zero xanthine oxidase activity, measured manometrically, contained a small amount of the enzyme when it was measured by its ability to form allantoin (11)

lized administered acetaldehyde at a somewhat slower rate than normal, but, like other such comparisons of uric acid formation (5) or the reduction of organic nitro groups (12), the effect *in vivo* was much less impressive than that *in vitro*

The rate of reduction of methylene blue when only DPN was added to the dialyzed homogenate was markedly decreased when the livers were obtained from rats fed tungstate or a protein-free diet (Experiments 3 and 5, Table I) It is believed that, under the experimental conditions used, this reduction of methylene blue by the addition of only DPN resulted from the action of xanthine oxidase on the hypoxanthine produced by the breakdown of DPN (11) The reasons for this belief are as follows (a) such a breakdown has been indicated (11), (b) the dialyzed homogenates should be relatively free of endogenous substrates which could act as electron donors to DPN and methylene blue, (c) the dye reduction was slow in those livers which were very low in xanthine oxidase, and (d) the rate of reduction of cytochrome c by DPNH with an ultracentrifuged supernatant fraction of the dialyzed homogenate was not affected by the molybdenum deficiency induced by tungstate feeding

SUMMARY

The diphosphopyridine nucleotide-linked acetaldehyde-oxidizing enzyme of rat liver was found to be independent of dietary molybdenum The aldehyde-oxidizing activity of a dialyzed liver homogenate without added diphosphopyridine nucleotide constituted only 15 to 20 per cent of the total activity and was Mo-dependent Feeding a protein-free diet caused the elimination of 90 per cent of the former and all of the latter activities

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THE PENTOSE CYCLE AS A PATHWAY FOR GLUCOSE METABOLISM IN INTACT LACTATING DAIRY COWS*

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The classical scheme of Embden-Meyerhof has been generally accepted as describing the pathway for glucose metabolism in animals, although recent observations have challenged this tenet. A number of *in vitro* studies based on the differential conversion of specifically labeled glucose to CO_2 have indicated the existence of a pathway in addition to the Embden-Meyerhof pathway for glucose metabolism in several animal tissues. Evidence for an alternate pathway has been obtained with rat liver (1-7) and mammary gland (8, 9), mouse liver (6), and rabbit spleen, testis, and bone marrow (10). Estimates made by various investigators have indicated that in liver slices almost 0 to as much as 50 per cent¹ of the glucose may be metabolized by an alternate pathway (3, 4, 6, 7). In rat mammary gland slices, it was estimated that approximately 60 per cent of the glucose was metabolized along an alternate pathway (8).

In vitro studies with rat muscle (1, 2) and with rabbit brain (10) have indicated that the Embden-Meyerhof pathway accounts for essentially all of the glucose metabolized in these tissues. A similar conclusion has been reached in studies with intact rats (1, 12, 13). The discrepancy between results obtained with the intact rat and rat tissues either indicated an artifact under *in vitro* conditions or showed that in the intact animal little glucose is metabolized in those tissues which have an alternate pathway for glucose metabolism (1).

Recently Bloom *et al.* (14) detected the pentose cycle in intact non-lactating rats, based on labeling patterns in glucose after administering specifically labeled ribose and glucose, however, they made no estimate of the quantitative significance of the pathway.

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¹ See the review by Wood (11) in which he discusses the calculations made by various investigators and the uncertainties in their assumptions which account, in part, for the variation between results.

Earlier studies with the intact cow demonstrated that glucose was rapidly metabolized, and within 35 hours essentially all of an injected dose appeared in the respiratory CO_2 (40 per cent) and milk constituents (56 per cent) (15). The nature of the pathways involved in glucose catabolism was not apparent in these studies since the injected material was uniformly labeled.

The present paper reports the results of trials in which two lactating cows were injected intravenously with glucose-6- C^{14} and then, after several weeks, with glucose-1- C^{14} . The transfer of C^{14} from glucose to CO_2 , to glycerol of milk fat, and to alanine and serine of casein could not be explained by exclusive operation of the Embden-Meyerhof pathway. Estimates based on C^{14} recovery in CO_2 and various milk constituents, in each case, indicated that the pentose cycle or a similar pathway played a significant role in glucose catabolism of the intact lactating cow.

TABLE I
*Data on Experiments with Cows Injected Intravenously
with Glucose-1- C^{14} and Glucose-6- C^{14}*

Trial No	Cow No	Body weight	Compound injected		Milk yield	Stage of lactation
		kg		mc	kg per day	wks
III	965	594	G-6- C^{14}	2 02	10	22
IV	965	611	G-1- C^{14}	1 80	9	27
V	84	423	G-6- C^{14}	1 96	12	7
VI	84	425	G-1- C^{14}	1 78	10	10

EXPERIMENTAL

Cows—Table I summarizes the characteristics of the two lactating Jersey cows used in these studies. Each cow served as its own control by being injected first with glucose-6- C^{14} (G-6- C^{14}) and, after 5 weeks (Cow 965) or 3 weeks (Cow 84), with G-1- C^{14} . In all trials 75 to 98 per cent of the C^{14} was accounted for in the respired CO_2 and milk constituents during the first 34 hours of the trial. Within a few days after G-6- C^{14} was injected, the C^{14} level in milk was too low to be detected by our counting equipment and it can be assumed that the amount of C^{14} remaining in the cows at the time of the second experiments, with G-1- C^{14} , was negligible.

Isotope—The C^{14} -labeled D-glucose was obtained from the National Bureau of Standards². The sugars were radiochemically pure, as shown by autoradiograms of samples chromatographed with butanol-acetic acid-

² The authors wish to express their appreciation to Dr H S Isbell, National Bureau of Standards, for his valuable cooperation in preparing millicurie quantities of G-1- C^{14} and G-6- C^{14} for use in these studies.

H₂O (4 4 1) and phenol-H₂O (10 4) The amount of C¹⁴-glucose injected into each cow is shown in Table I

Samples—Respired CO₂ was collected continuously during the first 3 hours of each trial and then at intervals until 34 hours The methods used for the collection and C¹⁴ assay of CO₂ have been described (16) Milk was collected periodically during the first 34 hours after isotope injection and was fractionated into its major organic constituents Casein was precipitated from the skim milk by adjusting the pH to 4.6 with 1 N HCl The casein was filtered, washed with water, and then redissolved in 1 N NH₄OH This procedure was repeated and the casein, after the third precipitation, was washed thoroughly with water, followed by alcohol and ether 5 gm. of dried casein were hydrolyzed, and the alanine, serine, and glutamic acid were recovered separately from ion exchange columns and prepared in crystalline form by methods already described (17)

Glycerol was recovered from the aqueous phase that separated upon acidifying milk fat hydrolysates It was purified by preparing the tribenzoate derivative according to the method described by Mulliken (18)

Other methods used in this investigation have already been described, including those for combustion of samples and radioassay of the resulting CO₂ (17) Sakami's method was used for the stepwise degradation of serine (19)

Results

Fig. 1 shows the specific activity in the expired CO₂ as a function of time after injecting cows intravenously with G-1-C¹⁴, G-6-C¹⁴, and uniformly labeled glucose (G-U-C¹⁴) After each trial with G-6-C¹⁴, the specific activity of expired CO₂ increased much more slowly and its maximum was only one-half that of the trials in which G-1-C¹⁴ was injected, furthermore, the maximum for G-6-C¹⁴ occurred later (1 to 1.5 hours) than that for G-1-C¹⁴ (0.5 to 0.8 hours) These results demonstrate that C-1 of glucose was oxidized more rapidly than C-6 and indicate that these 2 carbon atoms, in part, followed different metabolic pathways

The results obtained with G-U-C¹⁴ have been included for comparison (15) In general the rate of appearance of C¹⁴ in CO₂ for G-U-C¹⁴ was intermediate between the results obtained with G-1-C¹⁴ and G-6-C¹⁴

The specific activities of serine and alanine from casein and of glycerol from milk fat are listed in Table II for different times after injection of G-1-C¹⁴ and G-6-C¹⁴ For each compound the specific activity was greater, in some samples by as much as two times, after G-6-C¹⁴ than it was after G-1-C¹⁴ Thus, the data from milk products, like those of expired CO₂, indicate that C-1 and C-6 of glucose do not follow a common metabolic pathway and that there is some mechanism in addition to the Embden-Meyerhof pathway for glucose metabolism in the cow

The mean specific activity in alanine (during 34 hours) was 50 per cent greater after G-6-C¹⁴ than it was after G-1-C¹⁴ for both cows (see Table V)

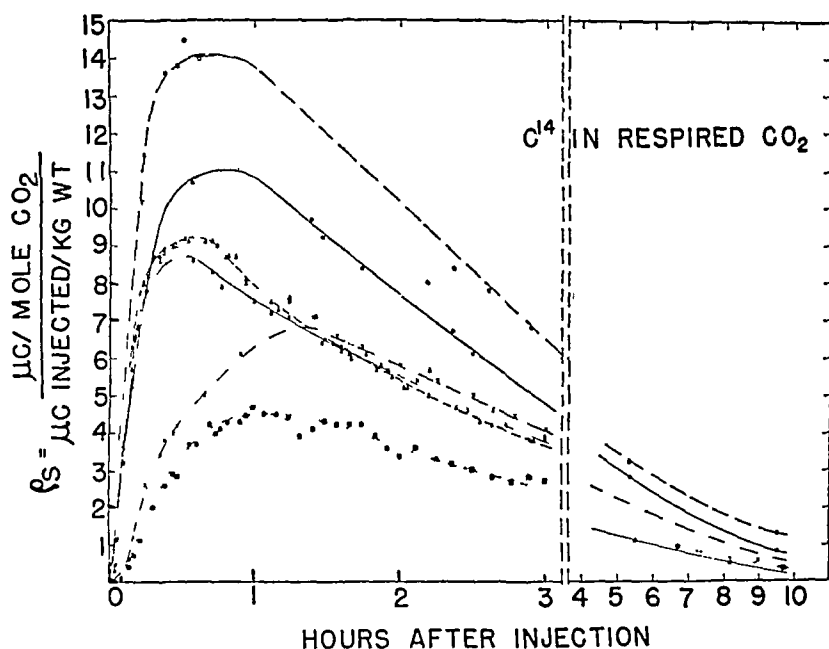


FIG 1 Standardized activity of respired CO₂ from cows injected with glucose-C¹⁴. The lines have been sketched in to show the trend of the experimentally determined values which are represented by the characters on Fig 1 ○, glucose-1-C¹⁴ (Trial IV), ●, glucose-1-C¹⁴ (Trial VI), △, glucose-U-C¹⁴ (Trial I), ▲, glucose-U-C¹⁴ (Trial II), ×, glucose-6-C¹⁴ (Trial III), ■, glucose-6-C¹⁴ (Trial V)

TABLE II

Specific Activities of Milk Constituents after Injecting G-1-C¹⁴ and G-6-C¹⁴*

After injection <i>hrs</i>	Glycerol			Serine			Alanine		
	$\lambda_s \dagger$		Ratio, 6 1	$\lambda_s \dagger$		Ratio, 6 1	$\lambda_s \dagger$		Ratio, 6 1
	G 1 C ¹⁴	G 6 C ¹⁴		G 1-C ¹⁴	G 6 C ¹⁴		G 1 C ¹⁴	G 6 C ¹⁴	
3 3	5 0	12 8	2 6	7 7	19 3	2 5	11 0	19 1	1 7
9 3	10 9	26 4	2 4	3 6	7 3	2 0	6 0	8 4	1 4
23 5	3 9	8 6	2 2	0 6	1 6	2 7	1 2	1 6	1 3
34	1 3	3 8	2 9	0 5	0 6	1 2	0 6	1 1	1 8

* The data in Table II are from Trials III and IV

† λ_s = microcuries per gm atom of C per microcurie injected per kilo of body weight

In contrast to these results, the Embden-Meyerhof pathway would lead to the same specific activity in alanine (derived from pyruvate) for both

types of labeled glucose unless the trioses formed at the aldolase stage had different metabolic fates. For example, if the equilibration between phosphoglyceraldehyde and phosphodihydroxyacetone had been slow and the latter (representing C-1,2,3 of glucose) had been preferentially diverted into the pathway of glycerol synthesis, the observed labeling of alanine might be expected. However, such an explanation would not account for the greater recovery of C¹⁴ in expired CO₂ after G-1-C¹⁴. Furthermore, this interpretation of the results obtained with alanine would lead one to expect the C¹⁴ levels in glycerol to be higher from G-1-C¹⁴ than from G-6-C¹⁴ when, to the contrary, these levels were, in fact, 2 to 3 times as great after G-6-C¹⁴ as they were after G-1-C¹⁴ (see Table II).

Utilization of Glucose for Biosynthesis—The observed results with expired CO₂ and milk products can be explained by the combined operation of the Embden-Meyerhof pathway and an alternate pathway which, for purposes of calculation, we have assumed to be the pentose cycle. Our reasons for deciding that the pentose cycle functions as the alternate pathway are discussed later. To assess the relative importance of the pentose cycle we have assumed that it functions together with the Embden-Meyerhof pathway to provide the major mechanisms for glucose catabolism in the cow. In addition, we assume that pyruvate is formed at equal rates from C-1 and C-6 of glucose along the Embden-Meyerhof pathway but arises only from C-6 of glucose along the pentose cycle since C-1 is lost as CO₂ (Fig. 2). Alanine indicates the C¹⁴ level in pyruvate, since it can be formed from the latter by transamination. The amount of C¹⁴ in alanine would be the same after G-1-C¹⁴ and G-6-C¹⁴ (per unit C¹⁴ injected and per liter of milk produced) if the Embden-Meyerhof pathway operated exclusively. Concurrent glucose metabolism in the pentose cycle would result in greater transfer of C¹⁴ to alanine after G-6-C¹⁴, and the difference between results obtained with the two types of labeled glucose would be directly proportional to the quantity of glucose metabolized via the pentose cycle.

Under these conditions our estimate for the quantitative significance of the pentose cycle was derived³ for the data obtained with alanine (these data are listed in Table III) as shown below: in Trials III and IV, $100 \times (0.0321 - 0.0178)/0.0321 = 44.5$ per cent via pentose cycle, in Trials V and VI, $100 \times (0.0365 - 0.0231)/0.0365 = 36.7$ per cent via pentose cycle.

The results from the two cows are in close agreement and indicate that at the site of alanine synthesis about 40 per cent of the glucose molecules had been converted to pyruvate via the pentose cycle. If considerably more glycerol were formed from the C-1,2,3 moiety of glucose than from

³ This is essentially the method applied by Abraham *et al.* to estimate pentose cycle activity during fatty acid synthesis in mammary gland (8).

the C-4,5,6 moiety along the Embden-Meyerhof pathway (the data in Table II suggest that it was not), then our calculated values for the pentose cycle would be too high. On the other hand, if pentose arising in the pentose cycle was utilized for nucleotide synthesis, it would have an oppo-

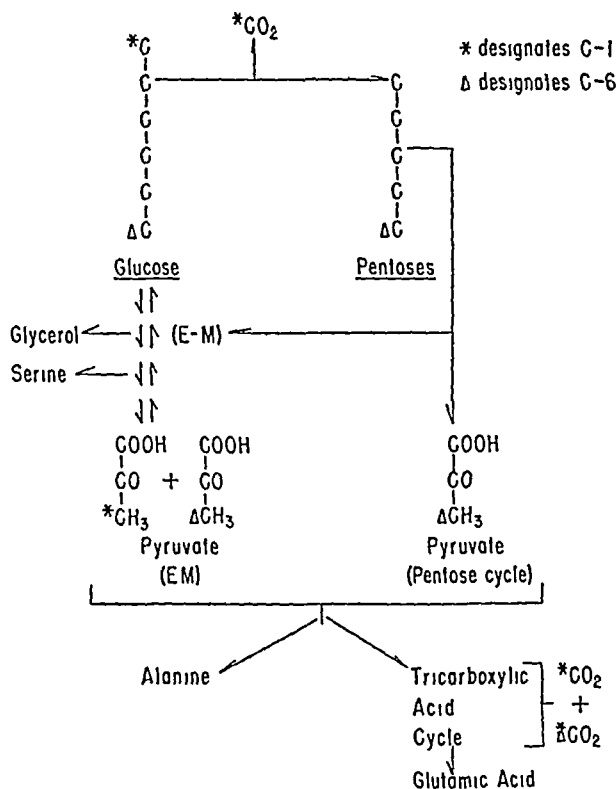


FIG 2 Hypothetical scheme of glucose metabolism along the Embden-Meyerhof (E-M) pathway and the pentose cycle. Two trioses arise via the E-M pathway, and it is assumed that these are converted to pyruvate in approximately equal quantities. In the pentose cycle, C-1 of glucose is oxidized to CO_2 and a triose is formed from C-4, 5, 6. This triose may mix with E-M intermediates at the phosphoglyceraldehyde or pyruvate level but, in either case, will result in the transfer of C^{14} to pyruvate only from C-6 but not C-1 of glucose. Pyruvate may give rise to alanine via transamination or may enter the TCA cycle, where it may be oxidized to CO_2 or converted into other compounds such as glutamic acid.

site influence and make our estimated values for the pentose cycle too low. Since we have no information on the relative magnitude of glycerol or nucleotide (pentose) synthesis from glucose, it is not possible to judge their influence on our calculated values.

Using the same method described above for alanine, we have estimated the quantitative importance of the pentose cycle based on the amounts of C^{14} recovered in serine and glycerol (Table III). The results of these cal-

culations are summarized in Table IV and indicate that 50 to 65 per cent of the glucose molecules were metabolized along the pentose cycle at the sites of synthesis of glycerol and serine

Glucose Oxidation—The C^{14} levels in respired CO_2 provide an additional

TABLE III
*Recovery of C^{14} in CO_2 and Milk Constituents during
34 Hours after Injection of Glucose- C^{14}*

Trial No	Position of C^{14}	Per cent injected C^{14} converted to			
		CO_2	Milk constituents*		
			Alanine	Serine	Glycerol
			$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$
III	G-6- C^{14}	33	3 21	5 68	37 5
IV	G-1- C^{14}	54	1 78	1 98	14 6
V	G-6- C^{14}	15	3 65	6 31	36 9
VI	G-1- C^{14}	49 7	2 31	2 74	18 2

* The per cent of injected C^{14} converted to milk constituents is expressed per liter of milk produced during the 34 hour period. This expression minimizes the influence of variations in milk production on the relative amounts of C^{14} transferred to milk constituents from G-1- C^{14} and G-6- C^{14}

TABLE IV
Quantitative Estimate of Glucose Metabolized via Pentose Cycle

Calculation based on C^{14} recovered per liter of milk in	Data from	
	Trials III and IV	Trials V and VI
	per cent	per cent
Alanine	44*	37
Serine	65	57
Glycerol	61	51

* The method for estimating the quantitative importance of the pentose cycle is discussed in the text

basis for estimating the quantitative importance of the pentose cycle. It is generally accepted that carbon from glucose is converted to pyruvate before oxidation in the tricarboxylic acid (TCA) cycle. Thus, it seems probable that the amount of $C^{14}O_2$ arising from G-1- C^{14} or G-6- C^{14} in the TCA cycle would be proportional to the mean specific activity of the pyruvate pool. If the alanine synthesized by the cow was derived from the same pyruvate pool that furnishes carbon to the TCA cycle, then the spe-

cific activity of alanine would also be proportional to the amount of $C^{14}O_2$ arising from G-1- C^{14} in the TCA cycle

As shown in Fig 2, CO_2 would arise from C-6 of glucose only in the TCA cycle but could arise from C-1 of glucose during metabolism in either the pentose cycle or the TCA cycle By using these conditions, the data from Trials III and IV provide the following indication of the importance of the pentose cycle In Trial III, after G-6- C^{14} was injected, the mean specific activity of alanine was 4.18 during the time that 33 per cent of the injected C^{14} was oxidized to CO_2 When the same cow was injected with G-1- C^{14} (Trial IV), 54 per cent of the C^{14} was oxidized to CO_2 , but the mean specific activity of alanine during this same period was only 2.84 From these data it may be estimated that $2.84/4.18 \times 33 = 22.4$ per cent of the C^{14} injected as G-1- C^{14} was oxidized to CO_2 by way of the TCA cycle in Trial IV⁴ Since 54 per cent of the C^{14} was recovered in CO_2 , it appears that $(54 - 22.4)/54 = 59$ per cent of the $C^{14}O_2$ from G-1- C^{14} arose via the pentose cycle The same calculations based on the data collected in Trials V and VI indicated that 80 per cent of C-1 of glucose was converted to CO_2 via the pentose cycle

Previous studies have demonstrated that, in the cow, glutamic acid of casein is derived from α -ketoglutarate in the TCA cycle (20) Thus the C^{14} level in C-1 of glutamic acid (Table V) should correspond closely to the C^{14} level of CO_2 arising in the TCA cycle From these data one can make an independent estimate of the relative importance of the pentose cycle in the oxidation of C-1 of glucose to CO_2 The method used for these calculations was the same as those discussed above for alanine, and the results are summarized in Table V The estimates calculated from the specific activities of glutamic acid C-1 are in close agreement with those based on alanine and indicate that 56 to 75 per cent of the CO_2 from glucose C-1 was formed via the pentose cycle

These values, which express the amount of CO_2 from C-1 of glucose formed via the pentose cycle, do not directly indicate the relative amount of glucose metabolized along this pathway, since the calculations neglected the amount of C^{14} utilized for biosynthesis along the Embden-Meyerhof and TCA cycle pathways In the pentose cycle, C^{14} from G-1- C^{14} is con-

⁴ For these calculations we assume that the relationship between alanine and the pyruvate pool and between the TCA cycle and the pyruvate pool is the same in Trials III and IV In other words, a given specific activity in the pyruvate pool (X) will result in the appearance of alanine in casein with a specific activity $A(X)$ where A is the dilution factor A will be approximately constant for a given cow on a given ration When the calculations are based on results collected over a 34 hour period, variations in A , due to fluctuations in food intake, tend to average out The same cow was used in Trial III and Trial IV and its ration was the same during both trials The same conditions apply for Trials V and VI

verted only to CO₂, whereas in the Embden-Meyerhof, TCA cycle pathway this C¹⁴ is converted to amino acids, fatty acids, glycerol, etc,⁵ as well as to CO₂. The amount of C¹⁴ entering these non-lactose milk constituents would be approximately equal to the total C¹⁴ injected as G-1-C¹⁴ minus the C¹⁴ in lactose and respired CO₂. In Trial IV, 54 per cent of the injected C¹⁴ was in CO₂ and 40.7 per cent in lactose, thus, 5.3 per cent can be assumed to have entered glycerol, fatty acids, amino acids, etc. In Trial VI the corresponding values were 49.7 per cent to CO₂ and 41.4 per

TABLE V
CO₂ from Glucose C-1 Arising via Pentose Cycle

The calculations summarized in Table V were based on the mean specific activities ($\bar{\lambda}_s$) of alanine and the C-1 of glutamic acid during the 34 hour experimental period

Trial No	Alanine		Glutamic acid C 1	
	$\bar{\lambda}_s^*$	Glucose C 1 to CO ₂ via pentose cycle	$\bar{\lambda}_s$	Glucose C 1 to CO ₂ via pentose cycle
		<i>per cent</i>		<i>per cent</i>
III	4.18	59	1.78	56
IV	2.84		1.28	
V	3.74	80	1.05	75
VI	2.48		0.865	

* $\bar{\lambda}_s = \frac{1}{34} \sum_0^{34} \lambda_s \Delta t$, where λ_s is the specific activity in microcuries per gm. atom of C per microcurie injected per kilo of body weight. The specific activity of the milk constituent from each milk sample was multiplied by the time period of milk formation, and the resulting products summated for the 34 hour experimental period.

cent to lactose, leaving 8.9 per cent to go into compounds other than lactose.

In Trial IV we estimated that 41 per cent (38 to 44 per cent, Table V) of the CO₂ from glucose C-1 arose in the Embden-Meyerhof, TCA cycle pathway which would be equivalent to 22 per cent (0.41×54 per cent) of the injected C¹⁴. An additional 5.3 per cent of the C¹⁴ went into compounds synthesized from the Embden-Meyerhof pathway and TCA cycle intermediates. Thus it appears that 27.3 per cent ($22 + 5.3$ per cent) of the injected glucose followed the Embden-Meyerhof pathway, whereas 32

⁵ It is assumed that most of the glucose incorporated into lactose was utilized without passing along either the Embden-Meyerhof pathway or the pentose cycle. Degradation of glucose from lactose shows that 95 per cent of the C¹⁴ was present in C-1 or C-6 when G-1-C¹⁴ or G-6-C¹⁴, respectively, was injected (Butterworth, E. M., unpublished data).

per cent was metabolized in the pentose cycle. Of the glucose catabolized by the two pathways, $32/59.3 = 54$ per cent followed the pentose cycle. The same methods applied to the data from Trial VI indicated that 65.7 per cent of the glucose catabolized entered the pentose cycle.

These values, based on CO_2 production from G-1- C^{14} , indicate that 54 to 66 per cent of the glucose catabolized entered the pentose cycle. They are in close agreement with the results based on C^{14} levels in milk constituents, which indicated that 40 to 65 per cent of the glucose catabolized had passed via the pentose cycle.

C^{14} Distribution in Serine—The distribution of C^{14} in the serine synthesized after injection of G-1- C^{14} and G-6- C^{14} is shown in Table VI. The

TABLE VI
 *C^{14} Distribution in Serine Recovered from Casein at
Intervals after Injection of G-1- C^{14} and G-6- C^{14}*

Trial No	Compound injected	Time of sample	C^{14} distribution		
			C-1	C-2	C-3
		hrs	per cent	per cent	per cent
IV	G-1- C^{14}	3	9	13	78
VI	"	3	8	12	80
VI	"	10	14	15	71
IV	"	34	20	24	56
III	G-6- C^{14}	3	3	13	84
V	"	3	3	11	86
V	"	10	4	9	87
III	"	24	7	13	80
V	"	34	10	15	75

C^{14} was located predominantly in C-3 of serine after injection of either type of labeled glucose. This labeling pattern excludes the Entner-Doudoroff scheme (21) and any similar mechanism from consideration as the alternate pathway in the cow. The Entner-Doudoroff pathway results in the conversion of C-1 of glucose to the carboxyl of pyruvate which would lead to greater labeling in C-1 of serine. The low C^{14} level in C-1 of serine also shows that CO_2 fixation was of minor importance in C^{14} transfer to serine and thus can be neglected without introducing serious errors in our estimation of pathways for glucose metabolism.

DISCUSSION

Isotope studies in intact animals seldom delineate pathways of metabolism but more often serve as a basis for deciding between various possibilities. In the present study with G-1- C^{14} and G-6- C^{14} , the results are

clearly inconsistent with exclusive operation of the Embden-Meyerhof pathway, although it is quite probable that this pathway accounts for a considerable part of the glucose catabolism in the cow. All of our results could be explained by postulating a combined operation of the Embden-Meyerhof pathway and pentose cycle in glucose catabolism in the cow.

Among the various alternate pathways proposed for glucose catabolism, the pentose cycle is the only one for which there is a substantial body of supporting evidence in animal tissues. Two enzymes of the pentose cycle, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are widely distributed in animal tissues and are found in high concentration in rat mammary gland (22). The levels of these dehydrogenases in rat mammary gland were found to increase 20- to 60-fold during lactation (23), which suggests a relationship between the pentose cycle and milk formation. These dehydrogenases have also been measured in sheep mammary tissue but apparently undergo smaller increases in activity during lactation than in rat mammary gland (9).

The disappearance of ribose 5-phosphate has been measured in several animal tissues *in vitro* and was found to be greatest in lactating mammary gland, especially during later stages of lactation (22). This measurement reflects, in part, pentose breakdown and indicates the presence of enzymes associated with the pentose cycle. Peeters *et al* (24) have detected a ketoheptose in colostrum and in mammary gland tissue from cows, which, according to chromatographic methods, appears to be sedoheptulose. For these reasons it appears most probable that a pathway similar to, if not identical with, the pentose cycle is responsible for part of the glucose metabolism in the cow.

The serine, alanine, and glycerol recovered from milk were probably synthesized to a large extent in liver and mammary gland, and the values calculated from their mean specific activities represent principally the influence of the pentose cycle on glucose metabolism in these organs. However, the quantitative significance of the pentose cycle estimated from alanine (40 per cent) was somewhat smaller than the estimate based on serine or glycerol (50 to 65 per cent).

One explanation for the lower result obtained in the case of alanine would be that alanine reflects a more general picture of glucose metabolism in the cow as a whole, whereas serine and glycerol reflect more closely metabolic pathways of a special organ such as mammary gland and liver. Studies *in vitro* indicate that, in muscle, glucose is metabolized predominantly, if not exclusively, by the Embden-Meyerhof pathway (2, 25). It seems probable that a part of the lactate produced in muscle mixes with lactate and pyruvate in liver (and mammary gland) and thereby modifies the specific activity of the pyruvate (and, in turn, alanine) produced in

those organs after injection of specifically labeled glucose. Under these conditions, the specific activity of alanine would be influenced by the pathway of glucose catabolism, not only in liver and mammary gland but in muscle as well.

Phosphodihydroxyacetone, the precursor of glycerol (26), and phosphoglyceric acid, the probable precursor of serine (27), arise at a higher level in the Embden-Meyerhof pathway than pyruvate and would not be expected to mix so largely with metabolites from other tissues, especially those from muscle.⁶ Under these conditions, the calculations based on the specific activities of glycerol and serine would represent more closely (than those based on alanine) the influence of the pentose cycle on glucose metabolism in liver and mammary gland. These values indicated that 50 to 65 per cent of the glucose had been metabolized via the pentose cycle. It is interesting that these values are in the same range as that derived by Abraham *et al.* (8) from *in vitro* studies with rat mammary gland, in which it was estimated that 60 per cent of the glucose had been metabolized by the pentose cycle.

Differences in the metabolic behavior of different parts of the body may decrease the validity of our assumptions used in making these calculations. For example, when the CO_2 produced from glucose metabolized along the Embden-Meyerhof pathway does not arise in the same TCA cycle as the α -ketoglutarate from which glutamic acid of casein is formed, then our estimate for the per cent C^{14}O_2 from glucose-1- C^{14} arising in the TCA cycle or pentose cycle may be in error. This problem arising from the inhomogeneity of the system is not necessarily avoided at the tissue slice or even single cell level, since the discrete distribution of enzymes within the cell may lead to metabolic pools that equilibrate with their surroundings at different rates.

The agreement between results calculated from five different sets of data and the agreement between results for the two cows lend strength to the conclusion that the pentose cycle plays a major role in the glucose catabolism of the lactating cow. It appears that at least 40 per cent of the glucose catabolism occurs via the pentose cycle, but in specific organs such as liver or mammary gland 60 per cent or more of the glucose may be catabolized via the pentose cycle.

SUMMARY

Glucose catabolism was studied with intact lactating dairy cows injected intravenously with glucose-1- C^{14} and glucose-6- C^{14} . The recovery of C^{14}

⁶ It is recognized that there may be some mixing between these compounds and pyruvate in liver and mammary gland during reverse glycolysis, but this would probably have a relatively smaller influence on the specific activities of serine and glycerol compared with the effect on alanine.

was greater in the expired CO_2 after glucose-1- C^{14} but was smaller in alanine, and serine from casein, and glycerol from milk fat, than the corresponding results after glucose-6- C^{14} . These results are inconsistent with the exclusive operation of the Embden-Meyerhof pathway and suggest an additional pathway of glucose metabolism through the pentose cycle.

The C^{14} levels in alanine, serine, and glycerol were used to estimate the quantitative importance of the pentose cycle in glucose catabolism. In each case the results indicated that one-half to two-thirds of the glucose molecules catabolized had entered the pentose cycle. Calculations based on C^{14} levels in respired CO_2 gave similar results. From these studies, involving assumptions, the limitations of which have been discussed in the text, we conclude that a non-Embden-Meyerhof pathway, which is probably the pentose cycle, has a major role in glucose metabolism in the lactating cow.

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CORRECTION

On page 262, line 3 from the bottom, Vol. 219, No. 1, March, 1956, read $[\alpha]_D^{27} - 12.1^\circ$
for $[\alpha]_D^{27} 12.1^\circ$

THE SOLUBILITY OF POWDERED BONE

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A number of attempts have been made in the past to establish a "solubility product" for bone (2, 6, 11), but there is little agreement in the literature on the results obtained. This opinion is ascribed (14) to the inconstancy of composition of the bone lattice due to its ability to "mirror the composition of its fluid surroundings." However, various workers have reported that, when bone is shaken with blood or biological fluids, there is a fall in the calcium and phosphate concentrations in the solution (2, 6), paradoxically indicating that tissue fluids are "supersaturated" with calcium and phosphate.

Neuman (13) showed that, when bone powder was shaken in a synthetic ultrafiltrate containing physiological concentrations of calcium and phosphate, it removed these ions from the solution in a 2:1 ratio until the product of their concentrations was about $0.6 \mu\text{mole per liter}$ at the end of 24 hours. The same bone sample appeared to have an almost unlimited capacity to take up calcium and phosphate in this way, the calcium and phosphate concentrations at the end of 24 hours always being about the same. It was not stated whether similar concentrations of calcium and phosphate could be obtained by starting from "undersaturation." For this reason the present attempt was made to establish a solubility product for bone powder.

Materials

The bone powder was prepared by grinding calf bone (with the epiphyses cut out) to about 100 mesh and then defatting and drying it with acetone and ether. All the experiments were performed on one batch of bone powder.

Procedure

In preliminary experiments it was found that the agitation of the bone powder in water and inorganic solutions resulted in the extraction of organic matter from the bone. Ultrafiltration of the supernatant fluid

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showed that a large but variable proportion of the calcium was not ultrafiltrable, it was presumably bound to protein or to polysaccharide extracted from the bone or to both. It was therefore decided to perform all the experiments with the powder in dialysis bags as Levinskas (10) had previously done in studies on the solubility of hydroxyapatite.

The standard procedure was to place a weighed amount of bone powder in a short strip of dialysis tubing with some of the equilibrating fluid and then to tie off both ends of the tube. Except where otherwise stated, the ratio of fluid to solid was kept constant at 10 ml of fluid to 1 gm of bone powder, this being the lowest practicable ratio and the one nearest to that existing in the body. In most of the experiments, 3 gm of bone powder and 30 ml of fluid were used, about one-third of the fluid being placed inside the bag and two-thirds outside. Equilibration was carried out in Erlenmeyer flasks which were mechanically agitated in an incubator at 37°. Bacterial growth was prevented by the addition of a crystal of thymol or, in later experiments, a few drops of toluene.

The equilibrating fluid used in most of the experiments is referred to as a synthetic ultrafiltrate and was made up as described by Neuman (13). It was brought to a pH of 7.4 with 5 per cent carbon dioxide. The tris-(hydroxymethyl)aminomethane (Tris) and cacodylate buffers were made up according to standard procedures at a strength of 0.15 M. When calcium or phosphate was omitted from or added to the synthetic ultrafiltrate, no attempt was made to correct the small change in molarity which resulted.

Calcium was estimated by titration with ethylenediaminetetraacetic acid in the presence of alkali, with ammonium purpurate as the indicator, the end point being established in a Coleman spectrophotometer. Phosphate was estimated by the method of Fiske and Subbarow (1) and pH measurements were made with a glass electrode pH meter.

Results

Experiments at pH 7.4 to 7.6—All of the results obtained by equilibrating the bone powder for periods of 16 to 72 hours at a pH of about 7.4 with synthetic ultrafiltrate and other buffered solutions of the same ionic strength containing various concentrations of calcium and phosphate are shown in Table I and Fig. 1. Table I shows the initial and final concentrations of calcium and inorganic phosphate, the initial and final pH (when measured), the nature of the equilibrating fluid, and the time of equilibration.

Most of these experiments were conducted for 24 hours. In the case of Experiment GG-2, in which the initial calcium level was very high, the 72 hour levels are given. Evidence that equilibrium was normally reached

in a few hours was obtained on several occasions, and the results after 5 hours in Experiment II are shown in Table II. These 5 hour levels are well within the range of the results shown in Table I. In other experiments, observations were continued for 72 hours, and it was found that only very small and variable changes occurred between 24 and 72 hours (Tables V and VII).

The change in pH which occurred in this pH range was small in contrast to the large changes which occurred in experiments at lower pH levels (Table III).

The final product of the calcium and phosphate concentrations in these experiments ranged from 0.28 to 0.74 $\mu\text{mole per liter}$. The mean product was 0.44 $\mu\text{mole per liter}$. When this figure is corrected to allow for the proportion of inorganic phosphate present in the divalent form at pH 7.4 in 0.15 M solutions (78 per cent) and for ionic strength effects with activity coefficients for calcium of 0.36 and for phosphate of 0.23,¹ the activity product becomes 2.8×10^{-8} mole per liter. This may be compared with an activity product of 2.65×10^{-7} for $\text{CaHPO}_4 \cdot \text{H}_2\text{O}$ ¹ and an activity product on dissolution of about 5×10^{-9} (10) for hydroxyapatite under comparable conditions.

To make these figures comparable with those of certain earlier workers (2, 6, 11, 12) they have also been expressed in terms of the pK of secondary and tertiary calcium phosphate. The solubility product, in these terms, of the secondary phosphate ranges from 6.2 to 6.7 and that of the tertiary from 24.7 to 26.7. When the experiments from supersaturation and undersaturation are considered separately, they do not show any significant difference in the range of either of these pK values.

Experiments at pH 6.2 to 7.8—When an attempt was made to establish the effect of pH on the solubility product, it was found that 0.15 M buffers were incapable of maintaining a constant pH at levels appreciably below 7.4. Table III gives some examples of the pH changes which occurred during equilibration. It was therefore found necessary to check the pH at frequent intervals and to bring it back to the desired pH by the addition of 0.15 M acid or base (usually the former). When this had been done frequently through the 1st day, it was found that there was less tendency for the pH to change thereafter, but no attempt was made to check it during the night, and in the mornings it was found that small changes had occurred.

The results obtained after 72 hours equilibration with this technique are shown in Table IV and Fig. 2. The effect of pH is very marked, the product varying from 3.9×10^{-6} mole per liter at pH 6.2 to 0.19×10^{-6} mole per liter at pH 7.8. It will be noticed that the line joining these

¹ Neuman, W. F., personal communication.

TABLE I

Solubility of Bone Powder at pH 7.3 to 7.6

The table shows initial concentrations in equilibrating fluid in all experiments carried out in the pH range 7.3 to 7.6, in which the ratio of solid to fluid was 1:10. All concentrations are expressed in moles per liter.

Experi- ment	Fluid	Initial levels							Time, hrs	Final levels							
		pH	Ca	P	HPO ₄ ' × 10 ⁻³	PO ₄ ''' × 10 ⁻⁹	pK Ca- HPO ₄	pK Ca- (PO ₄) ₂		pH	Ca	P × 10 ⁻³	HPO ₄ ' × 10 ⁻³	PO ₄ ''' × 10 ⁻⁹	pK s.p. Ca- HPO ₄	pK s.p. Ca- (PO ₄) ₂	Ca × P × 10 ⁻³
K-1	Synthetic ultrafiltrate	7.4	2.5	0.12	0.10	0.86	6.6	27.9	16	7.5	0.78	0.39	0.32	3.7	6.6	26.2	0.30
K-2	Synthetic ultrafiltrate	7.4	2.5	0.42	0.33	3.0	6.1	26.9	16	7.5	0.75	0.45	0.37	4.3	6.6	26.1	0.34
K-3	Synthetic ultrafiltrate	7.4	2.5	1.2	0.96	8.6	5.6	25.9	16	7.5	0.62	0.45	0.37	4.3	6.6	26.3	0.28
K-4	Synthetic ultrafiltrate	7.4	2.5	1.9	1.5	13.8	5.4	25.5	16	7.5	0.45	0.77	0.63	7.4	6.6	26.3	0.35
K-5	Synthetic ultrafiltrate	7.5	2.5	2.6	2.1	34.0	5.3	25.3	16	7.7	0.50	1.20	1.08	20.0	6.3	25.3	0.60
N-1	Synthetic ultrafiltrate	7.4	1.6	1.3	1.0	7.2	5.8	26.7	24	†	0.50	1.30	1.04	12.5	6.3	25.7	0.65
N-2	Synthetic ultrafiltrate	7.4	1.6	2.4	2.0	14.4	5.5	25.9	24	†	0.30	1.30	1.04	12.5	6.5	26.3	0.39
N-3	Synthetic ultrafiltrate	7.4	1.6	5.5	4.4	32.0	5.2	25.4	24	†	0.30	1.90	1.50	18.2	6.3	26.0	0.57
GG-2	Synthetic ultrafiltrate	7.4	27	1.2	0.96	8.6	4.6	22.9	72	†	6.0	0.10	0.08	0.96	6.2	24.7	0.60
II-2	Synthetic ultrafiltrate	7.4	0	1.3	1.04	9.4			24	7.5	0.20	1.70	1.40	16.4	6.6	26.7	0.34
II-3	Synthetic ultrafiltrate	7.4	0	1.3	1.04	9.4			24	7.5	0.20	1.80	1.50	17.3	6.5	26.6	0.36

II-4	Synthetic ultrafiltrate	7 4	1 8	1 3	1 04	9 4	5 7	26 3	24	7 5	0 39	1 30	1 04	12 5	6 4	26 0	0 51
II-5	Synthetic ultrafiltrate	7 4	1 8	1 3	1 04	9 4	5 7	26 3	24	7 5	0 25	1 20	0 96	11 6	6 6	26 7	0 30
JJ-4	Acetate	7 3	0 8	0 4	0 32	2 1	6 5	26 7	48	7 6	0 60	0 40	0 32	5 2	6 7	26 2	0 24
Y-4	Tris	7 4	0	0	0	0			24	7 4	0 60	0 50	0 40	3 6	6 6	26 5	0 30
QQ-3	"	7 4	0	0	0	0			24	7 4	1 50	0 28	0 24	1 8	6 4	26 0	0 42
RR-3	"	7 4	0	0	0	0			24	7 4	1 05	0 70	0 52	5 0	6 2	25 6	0 74
ZZ-3	"	7 4	1 5	2 2	1 8	13	5 6	26 2	24	7 4	0 90	0 56	0 45	4 0	6 4	25 9	0 50
UU-4	"	7 4	0	0	0	0			24	7 4	0 80	0 53	0 43	3 8	6 5	26 1	0 42

* S p = solubility product

† For the purpose of calculating phosphate fractions, the pH in these four experiments was assumed to be 7.5, although it was not measured.

points (the upper line in Fig 2) passes through pH 7.4 at a product very close to the mean value of $0.44 \mu\text{mole per liter}$ obtained in the previous

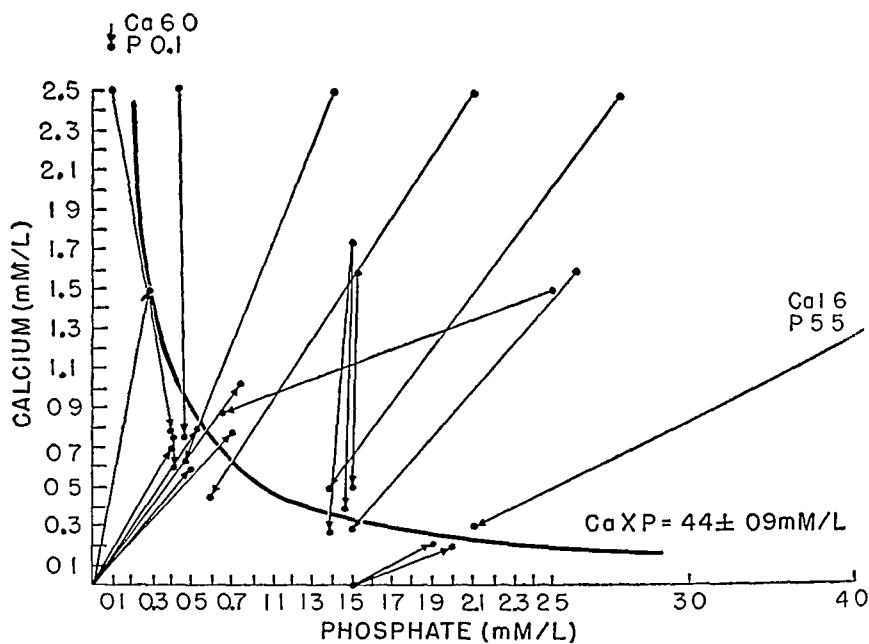


FIG 1 Solubility product at about pH 7.4. The black dots represent the concentrations of calcium and phosphate in the equilibrating solutions at the beginning of the experiments, and the tips of the arrows represent the concentrations at the end (usually 24 hours, see Table I). The line represents the mean final product of $0.44 \mu\text{mole per liter}$.

TABLE II
5 Hours Equilibration

The concentrations of Ca and P after 5 hours equilibration in Experiment II. See Table I for initial concentrations and 24 hour levels. All concentrations are expressed in moles per liter.

Experiment	Ca	P	Ca × P
	× 10 ⁻³	× 10 ⁻³	× 10 ⁻⁶
II-2	0.20	1.6	0.32
II-3	0.20	1.7	0.34
II-4	0.45	1.2	0.54
II-5	0.45	1.2	0.54

experiments. The lower line in Fig 2 (which is the product of the fifth and seventh columns in Table IV) shows that the effect of pH on the solubility product cannot be entirely explained in terms of the dissociation

of secondary phosphate ions, since the pK falls from 6.1 at pH 6.2 to 6.8 at pH 7.8. However, when calculated in terms of tertiary calcium phosphate, the solubility product is remarkably constant from pH 6.6 to 7.8, the extreme pK values being 26.1 and 26.7, the majority of them 26.4.

TABLE III

Changes in pH during Equilibration

Changes in pH of 0.15 M buffer solutions in the presence of bone powder

Experiment	Buffer	Initial pH	pH after	
			24 hrs	
KK-1	Acetate	5.4	6.7	
KK-2	"	5.6	6.9	
KK-3	"	5.8	7.1	
KK-4	"	6.0	7.1	
KK-5	"	6.2	7.3	
			12 hrs	48 hrs
NN-1	Tris-maleate	5.6	6.2	6.3
NN-2	"	5.8	6.5	6.5
NN-3	"	6.0	6.6	6.6
NN-4	"	6.4	6.9	6.9
NN-5	"	7.0	7.3	7.3
			24 hrs	
OO-1	"	6.9	7.0	
OO-2	"	7.1	7.0	
OO-3	"	7.3	7.4	
OO-4	"	7.5	7.6	
OO-5	"	7.7	7.7	
			48 hrs	
JJ-1	Acetate	4.3	5.5	
JJ-2	"	5.0	6.0	
JJ-3	"	6.0	7.0	
JJ-4	"	7.3	7.6	

The last three columns of Table IV show the product of the calcium and total phosphate concentrations at 24, 48, and 72 hours. The minor irregularities in the products between 24 and 72 hours may be due to failure to keep the pH absolutely constant despite the precautions taken. They certainly do not show any consistent trend which might suggest that equilibrium had not been attained.

Table V shows the results obtained at pH 6.2 and 7.8 when equilibrium was approached from higher values. The results should be compared

with those of Table IV in which all the equilibria were approached from below. In terms of the secondary salt, only a small difference can be seen between the results obtained by the two procedures, in experiments from above the pK range is 6.1 to 6.5, and in experiments from below, 6.1 to 6.8. In terms of the tertiary salt, the range in the undersaturation experiments is 26.1 to 27.3, and in the supersaturation experiments 25.4 to 27.5. In each series of experiments, the "solubility" of the secondary salt is greatest in the acid medium, and that of the tertiary salt greatest

TABLE IV
Effect of pH on Solubility

The effect of changes in pH upon the solubility of the bone powder. All solutions were free of calcium and phosphate at the beginning of the experiments. All concentrations are expressed in moles per liter.

Experiment	Fluid	pH	Time, hrs	Ca	P	HPO ₄ ''	PO ₄ '''	pK _{s.p.*} CaH- PO ₄	pK _{s.p.*} Ca ₃ (PO ₄) ₂	Ca × P		
				× 10 ⁻³			× 10 ⁻¹⁰			× 10 ⁻⁶		
										24 hrs	48 hrs	72 hrs
YY-1	Cacodyl buffer	6.2	72	4.0	0.97	0.19	0.85	6.1	27.3	4.6	3.5	3.9
YY-2	" "	6.4	72	3.9	0.76	0.21	1.52	6.1	26.9	3.2	2.2	3.0
YY-3	" "	6.6	72	3.5	0.46	0.17	2.10	6.2	26.7	1.6	1.3	1.6
YY-4	" "	6.8	72	2.7	0.46	0.22	4.50	6.2	26.4	0.93	1.0	1.3
YY-5	" "	7.0	72	2.3	0.39	0.23	7.80	6.3	26.1	0.53	0.63	0.7
QQ-1	Tris	7.0	72	1.8	0.31	0.18	6.20	6.5	26.7	0.42	0.50	0.56
QQ-2	"	7.2	72	1.3	0.33	0.23	13.0	6.5	26.4	0.50	0.34	0.42
QQ-3	"	7.4	72	0.7	0.49	0.39	35.0	6.6	26.4	0.65	0.45	0.36
QQ-4	"	7.6	72	0.4	0.63	0.54	82.0	6.7	26.4		0.29	0.25
QQ-5	"	7.8	72	0.25	0.75	0.69	170.0	6.8	26.3	0.26	0.24	0.19

* S p = solubility product

in the alkaline medium, but bearing in mind that the latter product involves a fifth order calculation, the results from below in the latter case appear extremely consistent.

Effect of Varying Solid to Fluid Ratio—In all the experiments described thus far, the solid to fluid ratio was 1:10. It was felt that if the results obtained represented any sort of solubility product, they should be reproducible at other solid to fluid ratios, at least within certain limits. Studies were therefore carried out with 100 mg, 500 mg, 1 gm, 3 gm, and 5 gm of bone powder, in each case with 50 ml of fluid. The results are shown in Table VI, which indicates that substantially the same results were obtained over the whole of this 50-fold range of solid to fluid ratio.

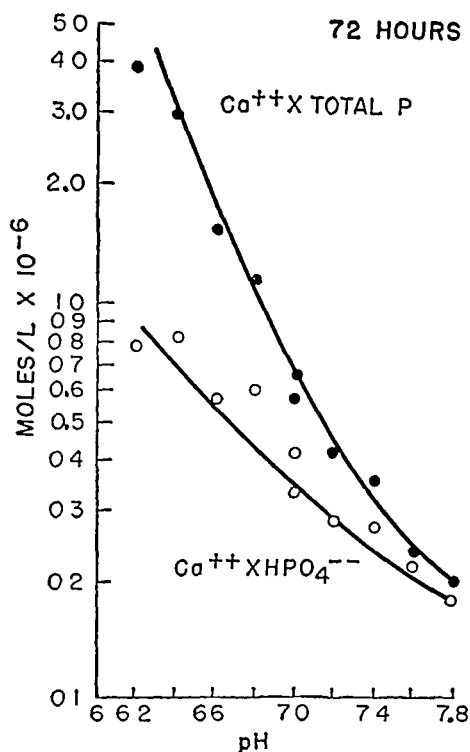


FIG 2 Effect of pH on solubility product The black dots show the product of the calcium and phosphate concentration after 72 hours equilibration at various pH levels The white circles show the corresponding figures after correction of total phosphate to divalent phosphate ions (see Table IV)

TABLE V

Equilibration from Above

Concentrations of calcium and phosphate after 24 hours equilibration with bone powder, starting from "supersaturated" solutions Tris buffer at pH 7.4 and 7.8, cacodylate buffer at pH 6.2 All concentrations are expressed in moles per liter

Experiment	Initial levels					Final levels (24 hrs)					
	pH	Ca	P	HPO ₄ '	PO ₄ '''	Ca	P	HPO ₄ ''	PO ₄ '''	pK _{sp} CaH PO ₄	pK _{sp} Ca ₃ (PO ₄) ₂
		× 10 ⁻³			× 10 ⁻³	× 10 ⁻³			× 10 ⁻³		
AAA-1	7 8	2 1	2 2	2 0	4 8	0 55	0 68	0 61	1 5	6 5	25 4
AAA-2	7 8	2 1	2 2	2 0	4 8	0 50	0 62	0 55	1 4	6 6	25 8
AAA-3	7 4	1 5	2 2	1 8	1 6	0 90	0 56	0 44	0 4	6 4	26 0
AAA-4	6 2	1 8	2 2	0 4	0 02	4 9	0 62	0 12	0 006	6 2	27 5
AAA-5	6 2	1 8	2 2	0 4	0 02	4 7	0 32	0 16	0 007	6 1	27 3

* Sp = solubility product

DISCUSSION

Neuman (14) and Hodge (3-5) have stressed that bone mineral is not a sufficiently pure or constant compound to possess a true solubility product. Nevertheless, since it has been formed in biological fluids of remarkably constant composition, it is not unreasonable that it should possess a relatively constant solubility, and it seems that the solubility of this

TABLE VI
Effect of Solid to Fluid Ratio

All samples were equilibrated with 50 ml of 0.15 M Tris buffer at pH 7.4. No calcium or phosphate in original solutions. All concentrations are expressed in moles per liter.

Experiment	Time	Solid	Ca	P	HPO ₄ ''	PO ₄ '''	pK _{sp} * CaHPO ₄	pK _{sp} * Ca ₃ (PO ₄) ₂
			× 10 ⁻³			× 10 ⁻³		
	<i>hrs</i>	<i>mg</i>						
UU-1	24	100	0.80	0.25	0.20	1.8	6.8	26.8
	48	100	0.80	0.22	0.18	1.6	6.8	26.9
	72	100	0.50	0.41	0.33	3.0	6.8	27.0
UU-2	24	500	0.80	0.41	0.33	3.0	6.6	26.3
	48	500	0.70	0.28	0.22	2.0	6.8	26.9
	72	500	0.65	0.44	0.35	3.2	6.6	26.6
		<i>gm</i>						
UU-3	24	1	0.80	0.44	0.35	3.2	6.6	26.3
	48	1	0.90	0.34	0.27	2.5	6.6	26.3
	72	1	0.88	0.44	0.35	3.2	6.5	26.2
UU-4	24	3	0.80	0.53	0.42	3.8	6.5	26.1
	48	3	0.90	0.59	0.46	4.3	6.4	25.9
	72	3	0.80	0.47	0.37	3.4	6.5	26.2
UU-5	24	5	0.80	0.52	0.41	3.7	6.5	26.2
	48	5	0.70	0.59	0.46	4.3	6.5	26.2
	72	5	0.80	0.44	0.35	3.2	6.6	26.3

* S_p = solubility product

particular sample of bone can be expressed in terms of the product of the calcium and phosphate concentrations with which it comes into equilibrium, under the conditions of these experiments. Whether similar results can be obtained on other bone preparations by other workers remains to be seen.

The figure obtained for the activity product of this bone preparation at pH 7.4 (2.8×10^{-8} mole per liter) is close to, but a little larger than, Levinskas' figure (10) for the solubility of synthetic hydroxyapatite in similar conditions, but smaller than the thermodynamic solubility product of dibasic calcium phosphate which is 2.65×10^{-7} (13). It is appreciably

smaller than the uncorrected product of the calcium and phosphate concentrations in mammalian tissue fluid, which is about 2×10^{-6} mole per liter (activity product about 1.3×10^{-7}). It is also considerably lower than the figure arrived at by Logan and Taylor (12) when they equilibrated very small amounts of inorganic solid or bone powder with very large volumes of calcium and phosphate solutions. They approached equilibrium from above and found that the solubility product became larger as the amount of solid used became smaller, until with very small amounts of solid the pK of the solubility product $\text{Ca}_3(\text{PO}_4)_2$ became 23.1. This figure is comparable with the product in the tissue fluids, and they believed that the lower figure obtained with larger amounts of solid could be explained by adsorption of ions on the surface. Another possible explanation might be that the small amounts of solid used were inadequate to take up the large amounts of calcium and phosphate present in the solutions. Logan and Kane (11) reported a number of calcium and phosphate values obtained by equilibration with bone, but in most cases the solid to fluid ratio was very much lower than that used in these experiments. However, with a solid to fluid ratio of 1:20 and a pH of 6.65, they obtained a calcium concentration of 2.55 and a phosphate concentration of 1.15 mmoles per liter. The product of these values is very close to the product obtained in the present experiments at this pH.

The solubility product for bone powder obtained at pH 7.4 is so far below the product in the tissue fluids even in hypoparathyroidism or vitamin D deficiency that it certainly cannot explain the levels of calcium and phosphate present in body fluids. However, a product comparable to that found in tissue fluid was obtained at a pH of about 6.6, and small changes in pH around this figure caused large changes in solubility, mainly due, presumably, to the large effect of pH changes in this region on the dissociation of phosphoric acid. These results suggest the possibility that the bone tissue in the living organism may be exposed to a bathing fluid at a lower pH than is normally assumed to be the case. It seems not inconceivable that a semipermeable "membrane" (as suggested by Howard (7)) or gel may separate the bone crystals from the tissue fluid and that a pH gradient may exist across this barrier. Possibly the interior of bone is not as well supplied with oxygen as other tissues, and the result is a local "acidosis" due to accumulation of CO_2 or organic acids. If such were the case, and this is, of course, mere speculation, parathyroid hormone and vitamin D would only have to effect a small additional lowering of pH locally in order to dissolve bone mineral (9). There has previously been speculation in this direction by Park (16) and Jaffe (8), among others.

The solubilities of calcium phosphate, bone powder, and synthetic hydroxyapatite have been compared in Table VII. In terms of secondary

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ORIGIN OF THE MAJOR SPECIFIC PROTEINS IN MILK*

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The proteins present in milk have been considered not only to arise from the synthetic activities of the mammary gland but also to include some preformed proteins which enter the gland from the blood and possibly other sources. The isolation and recognition in recent years of 97 per cent of the proteins of bovine skim milk as specific chemical and biological entities (1) have made it possible now to determine which of these proteins are synthesized in the mammary gland.

Various investigators (2-7) have shown with several species that intravenously injected radioactive precursors are incorporated rapidly into the milk proteins in the lactating mammary gland. Proteins synthesized in the gland and removed periodically by the milking process contain a notably higher level of activity up to 24 hours than proteins synthesized elsewhere and diluted by an existing protein pool. Black and Kleiber (5) have shown, with the aid of carbonate-C¹⁴, that in the cow in the early hours it is chiefly the non-essential amino acids which incorporate the activity. Campbell and Work (2) and Askonas *et al* (3) found with the rabbit and the goat that the total casein isolated from milk taken within a few hours after the intravenous administration of radioactive amino acids contains a higher level of activity than the total whey proteins. On the assumption that all of the casein was synthesized in the gland, this result was interpreted to indicate that some, at least, of the whey proteins must have a different origin. Part of the difference was accounted for by the immune globulins¹ which apparently enter the gland in a bound state from the blood, probably as the intact protein (6). Askonas *et al* (7) subse-

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¹ The immune globulins should not be confused with β -lactoglobulin. β -Lactoglobulin, α -lactalbumin, and milk serum albumin together comprise essentially the classical "lactalbumin" fraction of the whey proteins. The immune globulin components contain the antibodies of milk and comprise essentially the classical "lactoglobulin" fraction. The relation of the currently preferred nomenclature of the milk proteins to that of the past has caused some confusion. This has been clarified recently in a review by Jenness *et al* (1).

quently found in the goat that total casein and β -lactoglobulin are synthesized from a free amino acid pool in equilibrium with the free amino acid pool of the circulating blood. This implies that α -casein and β -casein are synthesized in the gland, since they comprise about 65 and 30 per cent, respectively, of the total casein.

It is possible from these studies to draw analogies among different species to arrive at conclusions concerning the probable origin of several of the major proteins of bovine milk. The present studies were conducted to establish for these and for the remainder of the major milk proteins which originate from the free amino acid pool in the gland and which enter the gland preformed from another source.

EXPERIMENTAL

A 1300 pound Holstein cow which was producing 24 pounds of milk per day in the 8th month of lactation was chosen for these studies. An injection of 20 mc of C^{14} as sodium carbonate was made into the right jugular vein. Blood and milk samples were taken before and at 3, 11, and 24 hours after injection. After an injection of 10 units of oxytocin, the cow was milked dry at each interval.

The milk samples were warmed to 37° , and the fat was removed by centrifugation. The skim milk was brought to pH 4.6 with dilute HCl and centrifuged. The precipitated casein was washed three times with distilled water and centrifuged, the first washing being added to the whey. The moist packed casein was stored at 4° under toluene until further fractionated. The whey samples were dialyzed against 4 volumes of distilled water for about 18 hours. These steps were carried out within 24 hours of the sampling time.

Casein Fractionation—The urea methods of Hipp *et al.* (8) were used with the following modifications. The α -casein precipitated from 4.63 M urea was washed three times with 4.7 M urea. The precipitate contained 90 per cent of α -casein contaminated with about 10 per cent of β -casein, as shown by electrophoretic analyses. The crude β -casein precipitated from 1.6 M urea was redissolved in 3.3 M urea, centrifuged, and precipitated by diluting to 1.6 M urea. The precipitate contained 93 per cent of β -casein contaminated with 5 per cent of α -casein and 2 per cent of γ -casein, as shown by electrophoretic analysis. The procedure for γ -casein was followed to the end of the second precipitation with ammonium sulfate. An electrophoretic analysis showed that this precipitate contained about 25 per cent γ -casein, 60 per cent β -casein, 5 per cent α -casein, and 10 per cent of an unidentified component with an electrophoretic mobility midway between α -casein and β -casein. This component accounted for less than 1 per cent of the original casein. Pure γ -casein was removed from the descending boundary of the electrophoretic cell with a hypodermic needle.

Whey Protein Fractionation—The dialyzed whey was clarified by centrifugation to remove traces of casein and lipoproteins and brought to pH 6.0 by the addition of neutral phosphate buffer. Two fractions were obtained by precipitation with solid ammonium sulfate at 2.06 M and 3.7 M.

The precipitate removed from the 2.06 M ammonium sulfate solution was dialyzed against distilled water and further fractionated by a modification of the method of Smith (9). The solution was adjusted to pH 4.6 with dilute HCl and a saturated solution of ammonium sulfate was added to give a 1.0 M solution. After centrifugation, the supernatant fluid was adjusted to pH 6.8 with dilute NaOH and saturated ammonium sulfate solution was added to give a 1.62 M solution. The precipitate was removed by centrifugation and contained about 85 per cent immune eu- and pseudoglobulins contaminated with 15 per cent of material with the electrophoretic properties of α -lactalbumin as shown by an electrophoretic analysis.

The precipitate removed from the 3.7 M ammonium sulfate solution was treated according to the procedures of Larson and Jenness (10) to prepare crystalline β -lactoglobulin. Some difficulty was experienced in crystallizing the β -lactoglobulin from some of the solutions after seeding, and in these instances the concentrated β -lactoglobulin solutions were dialyzed against distilled water after adjustment to pH 4.7 with acetate buffer. In either case, electrophoretic analysis showed that the precipitate contained 85 to 95 per cent β -lactoglobulin contaminated with α -lactalbumin. The supernatant fluid was adjusted to pH 4.0 with one-fourth its volume of 0.2 M potassium acid phthalate and brought to 1.0 M ammonium sulfate concentration by adding a saturated solution of this substance. Electrophoretic analysis of the precipitate showed that it contained about 95 per cent α -lactalbumin contaminated with β -lactoglobulin and milk serum albumin. The supernatant fluid contained about 20 per cent milk serum albumin, 70 per cent β -lactoglobulin, and 10 per cent α -lactalbumin. Pure milk serum albumin was removed from the ascending boundary in the electrophoretic cell by means of a needle.

Blood Protein Fractionation—Globulins were precipitated from the blood serum by raising the ammonium sulfate concentration slowly to 2.06 M with a saturated solution. The precipitated proteins contained 10 per cent of material with the electrophoretic mobility of blood serum albumin. The proteins in the supernatant fluid contained about 85 per cent of material with the electrophoretic mobility of blood serum albumin.

Preparation of Solutions for Analysis—The various preparations were dialyzed against water and neutral phosphate buffer to dissolve them and to remove all traces of urea and ammonium sulfate. An aliquot of 5 ml was removed and dialyzed three times against 20 volumes of phosphate buffer (pH 6.9, ionic strength of 0.1) for combustion and counting of the radioactivity. An aliquot of 20 ml was dialyzed three times against 30

volumes of Veronal buffer (pH 8.6, ionic strength of 0.1) for the electrophoretic analyses

Electrophoretic analyses were carried out as previously described (11, 12). Combustions for radioactivity assay were conducted by the wet method with use of the Van Slyke-Folch macro wet combustion fluid (13). The samples of CO_2 were precipitated as BaCO_3 and either counted at infinite thickness or, if not, corrected with a standard BaCO_3 self-absorption curve (14).

The results given for α -casein, β -casein, γ -casein, milk serum albumin, β -lactoglobulin, and α -lactalbumin are uncorrected since they were of 85 to 100 per cent purity and the contaminants were components with about the same level of activity. The immune globulins were corrected for the 15 per cent of material of high activity present, this had but a small effect on the result (*i.e.* 3 hour figures were corrected from 4.5 to 3.1 mc of C^{14} per mole of protein carbon).

RESULTS AND DISCUSSION

The protein analysis of the milk collected in this study is shown in Table I. The milk taken at the four intervals did not differ significantly in protein composition and was of the composition expected from a Holstein cow at this stage of lactation (15).

The results shown in Fig. 1 indicate that the total whey proteins incorporated less activity than the total casein. The level of activity found in the blood proteins was lower than that in the milk proteins, no significant difference was found between the levels incorporated into the blood albumin and globulin fractions. In all cases, the highest activity was found at the 3 hour level.

The curves in Fig. 2 show that the milk proteins may be separated into two distinct groups in terms of the activity incorporated. The level of C^{14} incorporated by α -casein, β -casein, α -lactalbumin, and β -lactoglobulin (hereafter called Group A) was about the same at each of the intervals, the highest level was present in the 3 hour milk. The level of activity incorporated into γ -casein, the immune globulins, and milk serum albumin (hereafter called Group B) was considerably lower at the 3 hour interval, increased a little at the 11 hour interval, and then decreased at 24 hours. The recognition that milk serum albumin as well as the immune globulins incorporate low activity adequately accounts for the difference in the level of activity incorporated into total casein and the total whey proteins.

These results imply that the conclusions reached by Askonas *et al.* (6, 7) for the synthetic origin of β -lactoglobulin and the immune globulins in the goat and rabbit also hold true for the cow. Since γ -casein comprises only a small percentage of the total casein (Askonas *et al.* noted that only a

trace was present in their total goat milk casein), it is apparent that the amount of activity incorporated into α -casein and β -casein may not differ significantly from that in the total casein

TABLE I
Protein Analysis of Milk

Protein fraction	Present per 100 ml skim milk
	gm
Casein (total)	2.96
α -Casein	1.98
β -Casein	0.83
γ -Casein	0.15
Whey proteins (total)	0.60
Immune globulins (eu- and pseudoglobulins)	0.07
α -Lactalbumin	0.12
β -Lactoglobulin	0.30
Milk serum albumin	0.03
"Minor" protein components*	0.08
Non-protein nitrogen (as protein)	0.21

* Contain enzymes, proteins unidentified or present in low concentrations, proteoses, and peptones, etc (1)

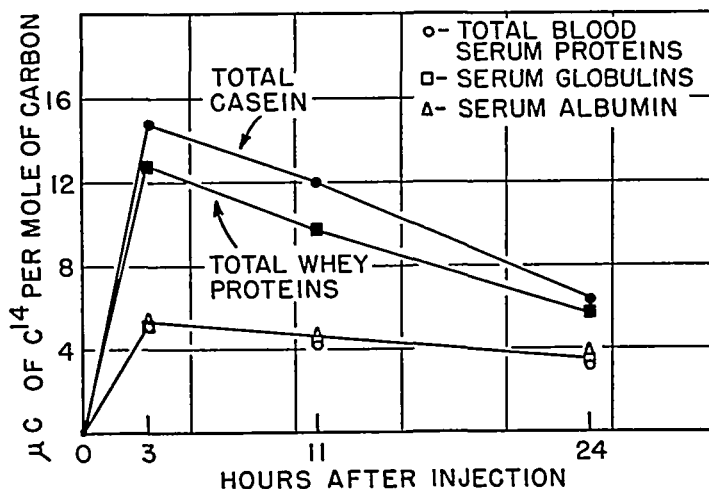


FIG 1 Level of C^{14} fixed in the total casein, total whey proteins, and the blood proteins after intravenous injection of $Na_2C^{14}O_3$

The similarity in the levels of C^{14} incorporated by each of the proteins in Group A suggests that these four proteins arise from a common origin. This similarity would be expected if these proteins were synthesized in the

gland from a common amino acid pool, since each of these proteins contains about the same level of the non-essential amino acids, especially glutamic and aspartic acids. If β -lactoglobulin, α -casein, and β -casein are synthesized in the mammary gland from a free amino acid pool, their level of incorporated activity represents the highest level that a protein could contain (within the limit of differences in amino acid composition). The fact that α -lactalbumin also incorporates the same high level implies that it must have been synthesized at the same time from the same free amino acid pool. This does not preclude the possibility that one or more of the four proteins in Group A could be synthesized elsewhere from the circulat-

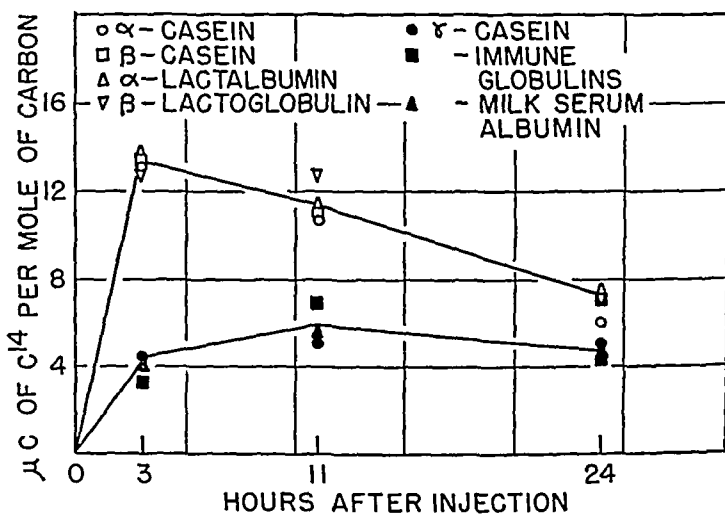


FIG. 2 Level of C^{14} fixed in the individual proteins of milk after intravenous injection of $Na_2C^{14}O_3$.

ing amino acid pool and be carried by the blood to the mammary gland. Such a situation is unlikely, since it would require that the mammary gland removed the protein from the blood stream so rapidly that it was not diluted by a pool of similar protein synthesized during the previous interval and present in the circulating blood. Attempts to isolate any of the proteins in Group A from the blood have proved fruitless and there has been no immunological evidence to indicate that they are ever present in the blood of either lactating or non-lactating animals (16). Furthermore, Heyndrickx and De Vleeschauwer (17) have observed in perfusion studies that the excised mammary gland continues to secrete milk with a normal or only slightly changed protein composition. The close relationship between the proteins in Group A also has been shown by previous work, which indicated that there is a highly significant correlation between the levels of the four proteins present in the milk of individual cows (12). Little rela-

tionship was found between the levels of the proteins in Group A and those in Group B

Any explanation for the origin of the three proteins in Group B must satisfy the data for radioactivity as well as the present day knowledge of protein synthesis and the physiological processes of an incorporation and excretion which occur in the mammary gland. The following hypotheses must be considered as possible explanations of the results observed for the proteins which incorporate low activity: (a) These proteins are synthesized in the mammary gland at the same sites as the other milk proteins but at a slower rate, (b) these proteins are synthesized in the mammary gland at a site separate from that for the other milk proteins and there is a lag between synthesis and transfer of the proteins to the site where they are incorporated as part of the milk, and (c) these components are blood proteins which migrate at a continuous rate from the blood stream (or lymphatic system) into the milk.

The first hypothesis may be eliminated since, if a protein were synthesized at the same site as the proteins in Group A, it must have originated from the same amino acid pool. The periodic removal of the synthesized protein by milking the cow does not allow a large pool of protein to accumulate. Hence, even if the synthesis were slower, the proteins formed during the interval at the same site should all contain about the same level of activity within the limits set by the differences in their amino acid composition. This assumes that the proteins are synthesized from a common free amino acid pool, an assumption which appears warranted from recent work with mammalian tissues (18). Even if one of the proteins in Group B were synthesized from peptide fragments or partially from the free amino acid pool, its level of C^{14} incorporation only fortuitously would be the same as the other proteins in Group B or of the blood serum proteins. Such a situation is unlikely.

The other two possibilities could equally well explain the activity curve with time and the lower specific activity of the proteins in Group B. The low value at the 3 hour interval is probably the result of dilution by a pool of unlabeled protein which arose either by synthesis and storage in the mammary gland or by migration of preformed proteins into the gland. In the one case, the diluting protein is that formed in the gland before injection of C^{14} but not released until the 3 hour milking time, when it would contain a fraction of high activity protein formed during the period, more of the high activity protein would be present in the 11 hour milking. In the other case, the constant migration of increasingly labeled blood protein into the milk would account for the low 3 hour value and for the increase at 11 hours. The drop at 24 hours occurs in all the protein fractions and is a reflection of general dilution and loss of C^{14} .

However, the close similarity in the specific activities of the blood proteins and the proteins in Group B suggests that they have a common origin. The level of activity found in the proteins of Group B at the milking intervals is what would be expected if the blood proteins were entering the gland during the intervening period. If this was not the case, it would have to be inferred that the ratio between the amount of highly active blood proteins synthesized in a given interval to the total amount of proteins in the blood stream was exactly the same as the ratio between the amount of a highly active protein synthesized in a given interval in the mammary gland to the total amount of this protein present in a pool in the mammary gland. Such a situation seems improbable. The high level of γ -casein found in the colostrum at parturition (15) and the observations of Heyndrickx and De Vleeschauwer (17) that this protein is similar in nature to the immune globulin components suggest a relationship of γ -casein to the blood proteins.

The available chemical and immunological data indicate that the immune globulins and milk serum albumin are identical with proteins present in the blood serum (1). These considerations suggest that the data for the proteins in Group B can best be explained by assuming that they are normal blood proteins which migrate into the milk from the blood stream. This does not eliminate the possibility that one or more of the proteins in Group B still could be synthesized in the mammary gland at a site other than the secretory tissue where the proteins in Group A are synthesized. Such a situation can be envisioned in the case of the immune globulins, for example, when synthesis of the various immune components occurs at several sites in the animal. One of these could be in the mammary gland. If the mammary gland is a site at which synthesis of a significant quantity of immune globulins occurs, however, the present data imply that they are not transferred to the milk directly but enter the blood stream first.

SUMMARY

20 mc of C^{14} (as Na_2CO_3) were injected intravenously into a lactating dairy cow.

The levels of C^{14} incorporated by α -casein, β -casein, α -lactalbumin, and β -lactoglobulin suggest that these milk proteins are synthesized in the mammary gland from a common free amino acid pool.

The levels of C^{14} incorporated by γ -casein, the immune globulins, and milk serum albumin, and their similarity with the levels present in the blood proteins, suggest that these proteins enter the milk preformed from the blood stream.

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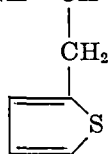
SYNTHESIS AND MICROBIOLOGICAL PROPERTIES OF A THIOPHENE ANALOGUE OF CARNOSINE*

By FLOYD W DUNN

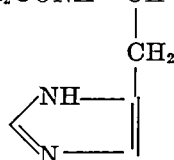
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Previous studies have shown that certain peptides of thienylalanine are effective growth inhibitors (1), and under some conditions the peptides are more toxic than thienylalanine (2). This paper reports the preparation of β -alanyl- β -2-thienyl-DL-alanine and studies of its effect upon the growth of *Escherichia coli* strain 9723. It can be seen from the structures below that this peptide analogue is structurally similar to carnosine (β -alanylhistidine), a naturally occurring dipeptide, and is also similar to β -alanylphenylalanine.



β -Alanyl- β -2-thienylalanine



β -Alanylhistidine

Since previous studies have demonstrated competitive antagonism between structurally similar peptides such as glycyl- β -2-thienylalanine and glycylphenylalanine (2), it might be expected that competitive antagonism would occur between the structurally similar β -alanyl peptides. It is shown in the experimental section that the toxicity of β -alanyl- β -2-thienylalanine is competitively reversed by the corresponding phenylalanine and histidine peptides, and the competitive relationships observed for the β -alanyl peptides appear to be functions of the peptides rather than the constituent amino acids.

EXPERIMENTAL

Compounds— β -Alanyl- β -2-thienyl-DL-alanine was prepared as described below, other compounds have been reported previously and were prepared by conventional methods or obtained commercially.

β -2-Thienyl-DL-alanine Methyl Ester Hydrochloride—10 gm of β -2-thienyl-

* This work was supported by grant No. RG-2843 from the United States Public Health Service, and was presented in part at the 130th meeting of the American Chemical Society at Atlantic City, September 16-21, 1956.

DL-alanine were esterified with methanol by a procedure similar to that described by Polglase and Smith (3) for other amino acids. A yield of 9.1 gm of ester hydrochloride melting at 160° was obtained.

$C_8H_{12}O_2NSCl$ Calculated, N 6.32, found, N 6.46

Carbobenzoxy- β -alanyl- β -2-thienyl-DL-alanine Methyl Ester—Carbobenzoxy- β -alanine (0.03 mole) was coupled with β -2-thienyl-DL-alanine methyl ester (0.03 mole) by the method of Vaughan (4) and others, as modified by Albertson and McKay (5), yielding 8.8 gm of product melting at 95°. The compound was purified by recrystallization from ethyl acetate-petroleum ether.

$C_{19}H_{22}O_5N_2S$ Calculated C 58.44, H 5.68, N 7.18
Found " 58.38, " 5.79, " 7.36

Carbobenzoxy- β -alanyl- β -2-thienyl-DL-alanine—5 gm of the above ester were hydrolyzed in 50 ml of acetone and 15 ml of 1 M NaOH at room temperature for about 1 hour. After removal of the acetone, the aqueous solution was extracted with ether, filtered, and acidified with dilute hydrochloric acid to precipitate carbobenzoxy- β -alanyl- β -2-thienyl-DL-alanine. Yield, 4.6 gm, after recrystallization from hot carbon tetrachloride, the melting point was 138°.

$C_{18}H_{20}O_5N_2S$ Calculated C 57.43, H 5.36, N 7.44
Found " 57.29, " 5.45, " 7.35

β -Alanyl- β -2-thienyl-DL-alanine—3.6 gm of carbobenzoxy- β -alanyl- β -2-thienyl-DL-alanine were allowed to react with 10 gm of 33 per cent HBr in glacial acetic acid by the method of Ben-Ishai (6), until evolution of CO₂ ceased. Upon addition of ether, a gummy precipitate of the peptide hydrobromide formed. After being washed with ether, the precipitate was dissolved in absolute ethanol and neutralized with concentrated ammonia to yield 1.6 gm of β -alanyl- β -2-thienyl-DL-alanine. After recrystallization from ethanol-water, the melting point was 255–257°.

$C_{10}H_{14}O_3N_2S$ Calculated C 49.57, H 5.82, N 11.56
Found " 49.42, " 5.94, " 11.45

Resting Cell Experiments—For studies to determine the ability of *E. coli* to hydrolyze the β -alanyl peptides, the cells were grown overnight in 300 ml of the salts-glucose medium (2). The cells were centrifuged, washed successively with saline and phosphate buffer (pH 7), and then suspended in 10 ml of phosphate buffer of pH 7. 1 ml of cell suspension, when combined with 1 ml of substrate solution, gave a final concentration of 2.25 mg of cells per ml. The tubes were incubated at 37° with occasional shaking. At intervals of 0, 60, 120, and 240 minutes, 30 μ l sam-

ples of the incubation mixture were withdrawn and placed on Whatman filter paper for preparation of the chromatograms. The samples were chromatographed by the ascending technique in 95 per cent methanol, and typical results are shown in Fig 1 for the 2 hour incubation mixture. The amino acids and peptides were detected by reaction with ninhydrin.

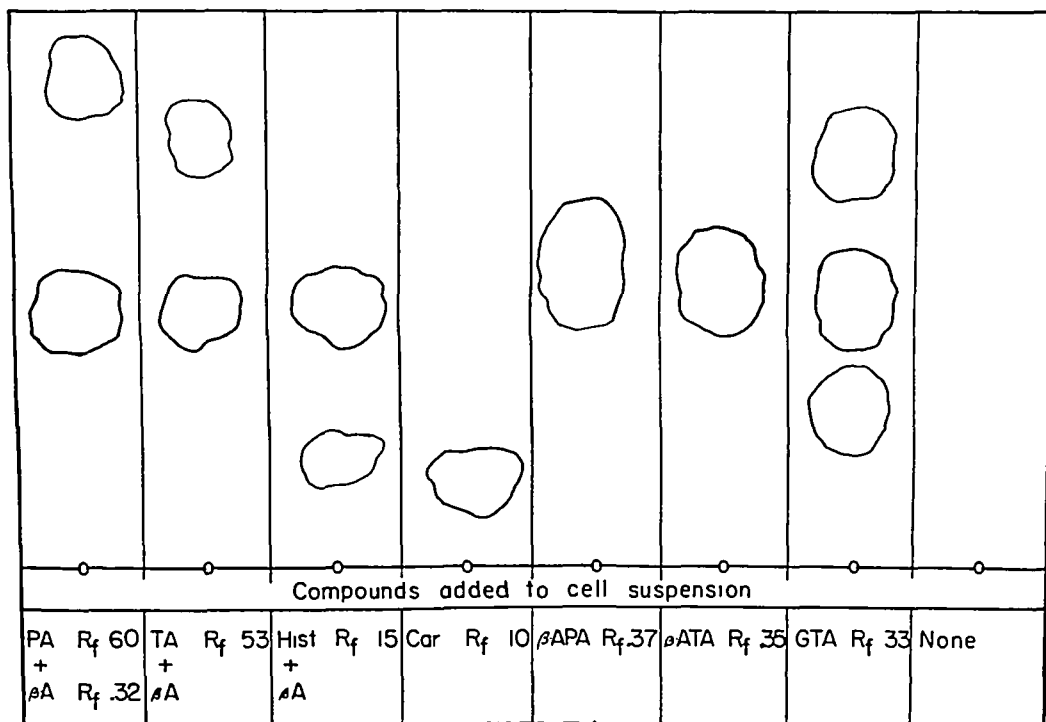


FIG 1 Drawing of typical chromatograms showing the failure of β -alanyl peptides to hydrolyze in the presence of resting cells of *E. coli* 9723. Glycyl- β -2-thienylalanine was partially hydrolyzed to form glycine (R_F 0.21) and β -2-thienylalanine (R_F 0.53). Abbreviations: PA, phenylalanine, TA, β -2-thienylalanine, β -A, β -alanine, Hist, histidine, Car, carnosine, β -APA, β -alanylphenylalanine, β -ATA, β -alanyl- β -2-thienylalanine, GTA, glycyl- β -2-thienylalanine.

Peptides were added to give a concentration of 1 mg per ml, whereas the free amino acids were added to give a concentration of 0.05 mg per ml.

Growth Studies—The testing procedure, employing *E. coli* strain 9723, was identical with that previously reported (2). The compounds were added aseptically to previously sterilized basal medium, and growth was determined by turbidity measurement with an instrument similar to that described by Williams *et al.* (7) in which distilled water reads 0 and an opaque object reads 100.

RESULTS AND DISCUSSION

Table I shows that growth of *E. coli* is completely inhibited with β -2-thienylalanine at a level of 1 γ per 5 ml. Reversal of the toxicity of β -2-thienylalanine (3 γ per 5 ml) is accomplished with phenylalanine (10 γ per 5 ml). However, even high levels of β -alanylphenylalanine, carnosine, histidine, or β -alanine were not utilized in reversing the effect of β -2-thienylalanine.

TABLE I

Effect of Phenylalanine, β -Alanylphenylalanine, Histidine, β -Alanine, and Carnosine upon Toxicity of β -2-Thienylalanine

Test organism, *E. coli* 9723, incubated for 14 hours at 37°

β 2 Thienylalanine	Amount of supplement*	Supplements†					
		None	PA	β APA	Hist	Car	β A
		Galvanometer readings‡					
γ per 5 ml	units per 5 ml						
0		65					
0.3		55					
1		1					
3	0		4	1	0	0	0
3	1		15				
3	3		55				
3	10		64				
3	100			2	1	0	0
3	300			0	0	1	0
3	1000			1	1	1	

* 1 unit of PA and β -APA contains the equivalent of 1 γ of DL-phenylalanine, and 1 unit of Hist and Car contains the equivalent of 1 γ of L-histidine.

† Abbreviations: PA, DL-phenylalanine; β -APA, β -alanyl-DL-phenylalanine; Hist, L-histidine; Car, L-carnosine; β -A, β -alanine.

‡ A measure of culture turbidity, distilled water reads 0 and an opaque object reads 100.

Table II shows that β -alanyl- β -2-thienylalanine causes appreciable inhibition of growth at a concentration of 10 units per 5 ml, although complete inhibition with this peptide does not occur even at 100 units per 5 ml. Phenylalanine and glycylphenylalanine were similar in reversing the toxicity of β -alanyl- β -2-thienylalanine non-competitively. β -Alanylphenylalanine was utilized competitively over a wide range of concentration in the reversal of β -alanyl- β -2-thienylalanine, giving an inhibition index¹ of about 30. Utilization of carnosine appeared to be competitive.

¹ The inhibition index is the ratio of inhibitor (β -alanyl- β -2-thienylalanine) to substrate (β -alanylphenylalanine or carnosine) required for a defined inhibition (11).

TABLE II

Effect of Phenylalanine, β -Alanylphenylalanine, Glycylphenylalanine, Histidine, Carnosine, and β -Alanine upon Toxicity of β -Alanyl- β -2-thienylalanine
 Test organism, *E. coli* 9723, incubated for 14 hours at 37°

β ATA	Amount of supplement*	Supplements†							
		None	Hist	PA	GPA	β APA	Car	β A‡	Hist + β A‡
		Galvanometer readings§							
<i>units per 5 ml</i>	<i>units per 5 ml</i>								
0		64							
3		48							
10		25							
30		17							
100		17							
30	0		16						
30	30		16						
30	100		16						
30	300		17						
0	3			64	64				
10	3			30	25				
30	3			25	20				
100	3			23	17				
0	10			64	66	68			
10	10					62			
30	10				38	41			
100	10			40	39	26			
300	10			40	33	20			
3,000	10			37	34				
0	30			64	67	67	65	64	
30	30					58	37	16	
100	30			63	55	46	23	14	
300	30			61	55	34	20	10	
1,000	30			62	55	19			
10,000	30			57	55				
0	100					68	67	62	63
30	100						60	14	19
100	100						35	10	17
300	100					57	21		11
1,000	100					28	13		
3,000	100					19			
0	300					68	67	64	63
30	300							16	22
100	300						44	14	19
300	300						30		11
1,000	300					46	16		
3,000	300					37			
10,000	300					13			
0	1,000						67		
100	1,000						58		
300	1,000						27		
1,000	1,000						17		

TABLE II—Continued

* 1 unit of β -ATA contains the equivalent of 1 γ of β -2-thienyl-DL-alanine, 1 unit of β -APA, GPA, and PA contains the equivalent of 1 γ of DL-phenylalanine, and 1 unit of Car and Hist contains the equivalent of 1 γ of L-histidine

† Abbreviations GPA, glycyl-DL-phenylalanine, β -ATA, β -alanyl- β -2-thienyl DL alanine, PA, DL-phenylalanine, Hist, L-histidine, Car, L-carnosine, β -A, β -alanine

‡ Separate experiments

§ A measure of culture turbidity, distilled water reads 0 and an opaque object reads 100

at lower concentrations of β -alanyl- β -2-thienylalanine, but, as the concentration increased, the inhibition index changed from a value of about 3 to a value of 1. Neither free β -alanine, histidine, nor an equimolar mixture of β -alanine and histidine showed any reversal of the toxicity of β -alanyl- β -2-thienylalanine.

Failure of β -alanylphenylalanine to reverse the toxicity of thienylalanine is in contrast to the behavior of other peptides of phenylalanine such as glycylphenylalanine, leucylphenylalanine, and alanylphenylalanine, which reversed thienylalanine non-competitively (2). It can be seen from Table I that as little as 1 γ of phenylalanine stimulates some growth in the presence of 3 γ of thienylalanine. Since 1000 units of β -alanylphenylalanine did not stimulate any growth in the presence of 3 γ of thienylalanine, it appears that the proteolytic enzymes of *E. coli*, under the testing conditions, do not hydrolyze the β -alanylpeptide to any significant amount.

Resting cell experiments were conducted to determine the ability of non-growing *E. coli* to hydrolyze β -alanyl peptides as compared with a glycyl peptide. Fig. 1 consists of a drawing of a typical chromatogram showing the failure of *E. coli* to split the β -alanyl peptides. After an incubation period of 2 hours there was no detectable spot for phenylalanine, β -2-thienylalanine, histidine, or β -alanine from the corresponding β -alanyl peptides, in contrast, there were definite β -2-thienylalanine and glycine spots after only 1 hour from the start of the hydrolysis of glycyl- β -2-thienylalanine. After 4 hours there appeared a faint β -alanine spot from carnosine, but there was still no evidence of phenylalanine or β -2-thienylalanine from the β -alanyl peptides. It seems obvious that the β -alanyl peptides, if hydrolyzed at all by *E. coli*, are hydrolyzed at a much slower rate than the glycyl peptides.

Since histidine and β -alanine, separately or in mixture, are not as effective as β -alanylhistidine in nullifying the toxicity of β -alanyl- β -2-thienylalanine, and since β -alanylphenylalanine reverses β -alanyl- β -2-thienylalanine, it appears that the competitive relationships observed represent reactions of the peptides as such rather than the constituent amino acids.

Such a behavior is in keeping with previous observations that peptides seem to have a metabolic function independent of prior conversion to the free amino acids (2, 8-10)

The author wishes to acknowledge the excellent assistance of Mrs Nancy Sloan and Mr Jack Davis in the chemical phase of this work and Miss Sara Spikes in the microbiological assay studies

SUMMARY

Preparation is described for β -alanyl- β -2-thienyl-DL-alanine and a number of intermediates employed in its synthesis. Growth studies employing *Escherichia coli* strain 9723 showed that this peptide was less toxic than β -2-thienylalanine, and that the toxicity was reversed non-competitively by phenylalanine and glycylphenylalanine. The toxicity of β -alanyl- β -2-thienylalanine was reversed in a fairly competitive way by the structurally similar carnosine and β -alanylphenylalanine. Histidine, β -alanine, carnosine, and β -alanylphenylalanine failed to nullify the toxicity of β -2-thienylalanine, histidine, β -alanine, and an equimolar mixture of histidine and β -alanine failed to nullify the toxicity of β -alanyl- β -2-thienylalanine. The results suggest that the peptides of β -alanine are utilized in some manner without prior conversion to the constituent amino acids.

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ISOLATION OF STEROIDS FROM A FEMINIZING ADRENAL CARCINOMA*

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The feminizing tumor of the adrenal gland occurs rarely in the male and is characterized clinically by gynecomastia, impotence, atrophy of the testes, and an increased excretion of estrogenic substances. In a recent review of the literature, Wallach *et al* (1) found that only twenty-two cases had been reported. From the urine of several of these, a number of substances related to dehydroepiandrosterone were isolated, but until the recent work of Landau *et al* (2), and Diczfalussy and Luft (3), the estrogenic steroids had not been identified. These workers found increased quantities of estradiol, estrone, and estriol, as well as pregnanediol, in the urine of their subjects.

While the urinary steroids might be assumed to reflect the secretion of the adrenal tumor, the degradative action of the liver and other tissues must be considered. The tissue analysis, therefore, was undertaken to study directly the precursors of the urinary products. This investigation resulted in the isolation of several steroids new to the human and contributed a possible method of the diagnosis of this tumor.

Materials and Methods

Tumor—The tumor weighing 920 gm was removed from the area of the left adrenal of a 22 year-old male patient of the Salt Lake City Veterans Hospital. A detailed discussion of clinical and other laboratory findings is published by Wallach *et al* (1). Pathological examination by Dr Oscar Rambo, Salt Lake City Veterans Hospital, and confirmed by the United States Armed Forces Institute of Pathology, revealed an adrenal carcinoma of the estrogen-secreting type, characterized by anaplasia, capsular vein invasion, and cords of cells strikingly "similar to those seen in granulosa cell tumors of the ovary."

Laboratory Methods—All solvents were purified by standard procedures.

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(4) and by distillation prior to use All evaporations involving more than 10 ml were carried out *in vacuo* When quantities less than 10 ml were to be evaporated, the solvent was blown off by a fine jet of nitrogen Acetylation was carried out in pyridine and acetic anhydride (1:3) at 100m temperature The Zimmermann reaction for the estimation of 17-ketosteroids was the routine clinical procedure in which *m*-dinitrobenzene in 2.5 N KOH in ethanol was employed

Extraction of Tumor—The portion of the tumor which was extracted chemically weighed 523 gm The frozen tissue was homogenized in a Waring blender and extracted three times with a total of 4 liters of acetone at 50° Two fractions were obtained the aqueous acetone fraction which

TABLE I
Chromatography of Acetone Extract on Silica Gel

Fraction No	Solvent	Volume	Weight of material	Comment
		<i>ml</i>	<i>mg</i>	
1	Hexane	100		Discarded
2	Hexane-benzene, 3:1	100	1	"
3	" 1:1	100	19	"
4	Benzene	100	25	Progesterone
5	Benzene-CHCl ₃ , 3:1	100	11	Nothing isolated
6	" 1:1	100	15	" "
7	CHCl ₃	200	1	Estrogens, other steroids
8	CHCl ₃ -CH ₃ OH, 1:1	200	1	Nothing isolated
9	CH ₃ OH	250	1	" "

was evaporated *in vacuo* and the protein residue which was hydrolyzed with 1 liter of 5 per cent NaOH for 2 days at room temperature

Acetone Fraction—The aqueous residue from the acetone fraction was extracted quantitatively with chloroform After the chloroform was removed *in vacuo*, the fatty residue was partitioned between hexane, which was discarded, and 75 per cent methanol, which was concentrated *in vacuo* The aqueous residue from the methanol was extracted with chloroform and dried for column chromatography The final weight of the yellow oil was 670 mg

Column Chromatography—The silica gel (100 to 130 mesh) was washed extensively with acetone and ether and activated by heating overnight at 100° A column 2 × 12 cm in dimension was prepared with 17 gm of silica gel in a hexane slurry The oil was placed at the surface by dissolving it in a small amount of chloroform-hexane solution, and the flask was rinsed many times with subsequent eluent solutions The details of the solvent system and residues are shown in Table I Only

the benzene fraction (Fraction 4) and the chloroform fraction (Fraction 7) yielded identifiable steroids

Reextraction of Tumor Residue after Alkali Hydrolysis—It is often difficult to extract steroids from crude tissue, and whether this is a reflection of "protein binding" or inadequate mechanical procedures is difficult to demonstrate. However, it is known that alkali digestion renders the material readily extractable with ether (5). It is often not desirable for a first step because of the degradative action of the alkali on the steroids. Progesterone and many estrogenic compounds, however, are stable to alkali, and for this reason the protein residue was hydrolyzed in 1 liter of 5 per cent NaOH for 48 hours at room temperature, extracted with ether at pH 10, and again extracted after acidification to pH 3. The dried residues of the individual extractions were not large, therefore, they were pooled, dissolved in ethyl acetate, and washed with saturated Na_2CO_3 at pH 9 according to the method of Brown (6). The extract was further purified by partition between hexane and 75 per cent methanol. The final pale yellow oil weighed 260 mg.

Paper Chromatography—Isolation of the individual steroids was accomplished by paper chromatography by the methods of Zaffaroni (7). Preliminary chromatography in the heptane-formamide system resolved the oil from the alkali digest into two gross components which were visible under an ultraviolet light scanner. The area at the origin was the source of the more polar steroids, and the area near the front yielded progesterone.

Criteria for Identification—The criteria for satisfactory identification of steroid structure are subject to controversy and are discussed in detail elsewhere (8). An R_F by itself carries little weight regardless of the number of times the chromatogram has been run. A single maximum in the ultraviolet absorption spectrum may often verify the presence of a grouping, but, when several peaks are obtained, the evidence for total structure is better. It is even more reliable if it can be shown that the coefficients of absorption (E_{max}) of the various maxima agree with the corresponding values of the authentic compound. In our experience, the sulfuric acid spectrum (9) is extremely reliable if several maxima are obtained, and the extinction coefficients of the maxima (E_{max}) are compared with those of the known compound. The infrared spectrum has unique value, but requires purity and quantities greater than were available from these extracts. In most cases we have attempted to make at least one derivative and preferably two.

Results

Progesterone—Progesterone was isolated and characterized both from the acetone extract and from the alkali digest. Fraction 4 from the silica

gel column was chromatographed on paper in the hexane-formamide system as was the progesterone area of the paper chromatogram of the protein digest residue. Both gave R_F values identical to those of a simultaneously run control on three separate occasions. By spectrophotometric

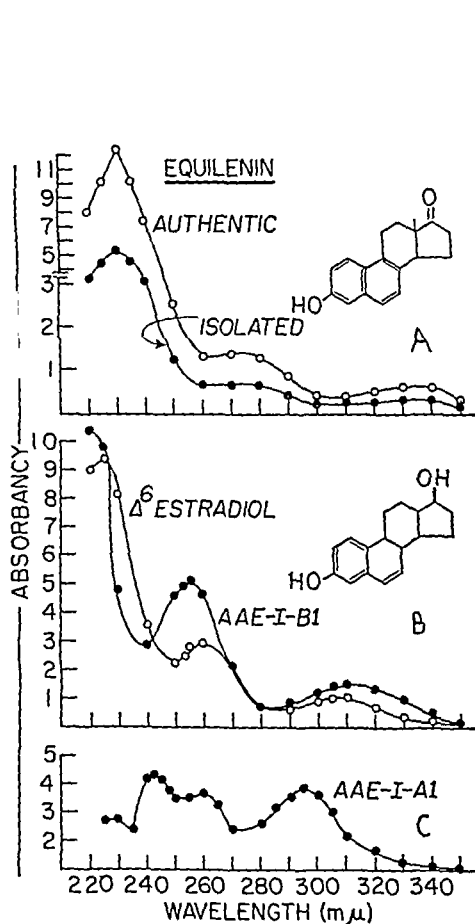


FIG 1

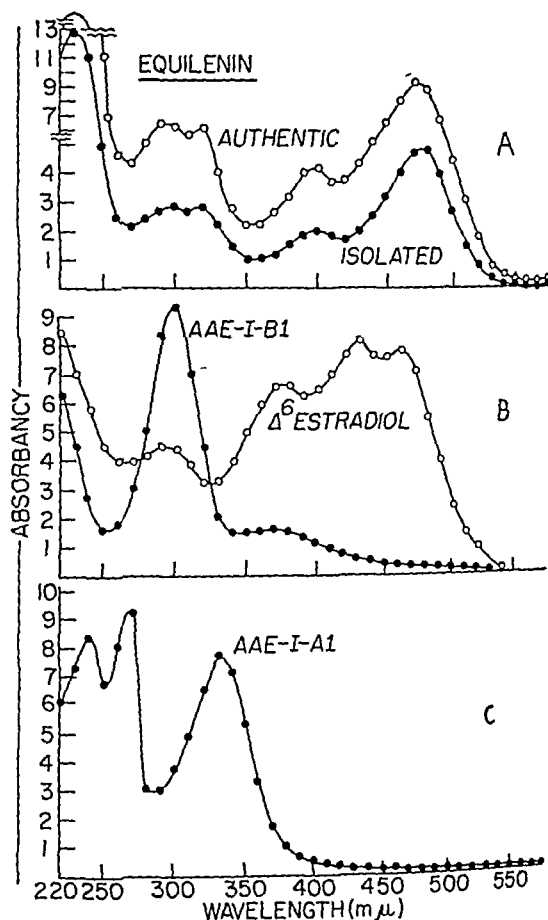


FIG 2

FIG 1 Ultraviolet spectra of the isolated phenolic steroids and authentic comparison substances in methanol

FIG 2 Chromogen spectra of isolated phenolic steroids and authentic comparison substances in sulfuric acid

analysis both had maxima at 240 $m\mu$, and the yields were 75 γ from the acetone extract and 400 γ from the hydrolysate. They were combined, and final identification was established by spectrophotometric analysis in fuming sulfuric acid and by infrared analysis. In both procedures the spectra were compatible with those of authentic progesterone.

Equilenin—Fraction 7 from the silica gel column (Table I) and the ori-

gm of the paper chromatogram of the alkaline extract were chromatographed in the benzene-formamide system for 20 hours. The mobility of this compound was identical to that of authentic equilenin. Spectroscopic absorption was maximal at 235, 280, and 340 $m\mu$ (Fig 1, A). Both the absorption maxima and the ratios of the extinction coefficients (E_{max}) at the maxima were equivalent to those of authentic equilenin. The test for the 3,5-unsubstituted hydroxyphenol group with Millon's reagent was positive (10). The Zimmermann reaction for the 17-ketone was positive, and the Zaffaroni chromogen (9) (Fig 2, A) gave maxima at 230, 300, 320, 400, and 472 $m\mu$, identical both in maxima and E_{max} with those of authentic equilenin. In concentrated sulfuric acid a green fluorescence was observed under long wave ultraviolet light similar to that obtained with au-

TABLE II
Substances Isolated from 523 Gm of Feminizing Adrenal Carcinoma

Products isolated	Acetone extract of tumor	Extract after hydrolysis with NaOH
	γ	γ
Progesterone	75	400
Equilenin	27	300
AAE-I-B1	44	345
AAE-I-A1	23	109
AAE-I-A2		93
AAE-I-C1		36
AAE-I-C2		339
AAE-I-D		50

thentic equilenin. From these data it was inferred that the compound was equilenin (Fig 1, A). The total amount isolated was 327 γ .

Compound AAE-I-B1 (Structure Not Identified)—Isolated from both acetone and alkaline extracts, this compound was slightly more polar than equilenin and at first appeared to be similar to 6-dehydroestradiol. It was overrun for 6 hours in benzene-formamide and traveled 9.5 cm identically with authentic 6-dehydroestradiol. The Millon test was positive. An acetate was formed, and this derivative had an R_F of 0.15, whereas the acetate of 6-dehydroestradiol did not move from the origin in this amount of time. The absorption maxima in methanol were seen at 220, 255, and 310 $m\mu$ as compared to those of 6-dehydroestradiol which occurred at 225, 260, and 305 $m\mu$ (Fig 1, B). The E_{max} ratios at those wave lengths were not comparable. The sulfuric acid chromogen yielded a sharp absorption maximum at 300 and a shoulder at 355 $m\mu$ (Fig 2, B). This confirmed the difference from 6-dehydroestradiol which gave sharp peaks

at 295, 370, 430, and 460 $m\mu$ (Fig 2, B) (The precariousness of deducing structure by ultraviolet absorption and R_F is reemphasized by the study of this compound) Temporarily we postulate from Woodward's rule (11) an additional conjugated double bond either endocyclic or exocyclic to the B ring, but we cannot define the remainder of the structure The total amount isolated was 389 γ

Other Substances—No substance isolated reacted positively with triphenyltetrazolium chloride Consequently, there was no evidence for the presence of the α -ketol side chain characteristic of the normal adrenal hormones A search for the expected phenolic estrogens failed to reveal the presence of estradiol, estrone, or estriol Other substances which were found but not identified are presented in Tables II and III in order of

TABLE III
Substances Isolated but Not Identified

Compounds	Millon test	Zimmermann reaction	Ultraviolet (MeOH) maximum	Chromogen (H ₂ SO ₄) maximum
			$m\mu$	$m\mu$
AAE-I-B1	+	—	220, 255, 310	300, 355
AAE-I-A1	+	+	242, 260, 295	240, 265, 330
AAE-I-A2	—		240, 300-325*	385
AAE-I-C1	—		240	
AAE-I-C2	—	—	240	280, 370, 435
AAE-I-D	—		240	

* Plateau

decreasing polarity Thus, AAE-I-A1 and AAE-I-A2 were slightly less polar than estriol and presumably were phenolic substances Substance AAE-I-D was slightly less polar than testosterone, but more polar than 4-androstene-3,17-dione

DISCUSSION

The previously published advantages of hydrolysis with sodium hydroxide prior to the extraction of steroids are reaffirmed in this work (5) While it would be difficult to postulate the cohesiveness of the steroid-protein "binding," certainly, the hydrolysis of the protein rendered the steroid more extractable Thus, the alkaline digestion of the acetone residue yielded 5 to 10 times more of each of the steroids than was obtained by acetone extraction, even though the acetone mixture was heated to about 50° (Table II)

It is impossible to interpret our results on the basis of other workers' findings Only about 2 dozen cases of "feminizing tumors" have been reported, and there is so much variation clinically, pathologically, and in

the urinary steroid analyses that comparison is not profitable. However, to our knowledge, this is the only feminizing tumor tissue from which steroids have been characterized and the only isolation of equilenin from a human source (12).

Equilenin was not the only unusual phenolic substance isolated, for analysis of compound AAE-I-B1 would certainly indicate a substance with a phenolic A ring and additional conjugated unsaturation. It could not be determined whether this unsaturation were endocyclic in the B ring or exocyclic, but the R_F would tend to support the former concept. This substance was neither 6-dehydroestradiol nor dihydroequilenin. The implication of finding equilenin and this substance in the human is that they are at least quantitatively unique to this malignancy and reflect either an aberrant aromatizing enzyme system or an overly active one. On the other hand, at least three different groups have found one or all three of the usual phenolic estrogens in the urine of their patients with feminizing tumors (2, 3), and Mason and Kepler (13) found estrone in the urine of a patient with adrenocortical hyperplasia.

Progesterone was the substance isolated in greatest quantity from the tumor. The amount isolated was as much as was found in term placenta (1 mg per kilo), but less than was found in sow ovaries (15 mg per kilo) (14). The normal ox adrenal, on the other hand, has been reported to contain 0.5 mg per kilo (15). Whether progesterone is present in the role of a precursor to the estrogens and other steroids (16-21) or whether the estrogens and progesterone are synthesized from common or different precursors is a consideration beyond the scope of this report. However, it is of interest that one is rarely found without the other.

On a clinical level, the isolation of progesterone is interesting because its presence in so many secretory tumors is reflected by increased levels of pregnane- $3\alpha,20\alpha$ -diol in the urine. Since this tumor occurs most often in males (1), who normally have low urinary levels of pregnanediol, the presence of increased amounts of the latter substance should certainly arouse clinical suspicion of endocrine malignancy. Thus, to cite a few examples, Landau *et al* (2) found elevated levels of pregnanediol in the urine of their patient, and Twombly (22) emphasizes the excretion of pregnanediol in cases of secretory ovarian tumors. If progesterone is a precursor to other steroids, its diagnostic role in cases of secretory tumors of the gonads and adrenals and their functional metastases deserves further investigation.

SUMMARY

Steroids were extracted and identified from a feminizing adrenal carcinoma. Preliminary hydrolysis of the tissue with sodium hydroxide was found to be 5 to 10 times more effective than extraction with warm acetone.

Progesterone and equilenin were isolated and identified, six other steroids were studied, but their structures could not be elucidated. There is evidence, however, for the presence of phenolic groups and other conjugated unsaturation in the molecule.

It is suggested that urinary study of pregnanediol and equilenin-like steroids would be of diagnostic value in patients suspected to have secretory tumors of this type. The significance of the findings in relation to endocrine tumors and their function is briefly discussed.

The authors wish to express their appreciation to Dr. Harold Brown and Dr. Stanley Wallach for the tumor and clinical information associated with this case, to Dr. Oscar N. Rambo for permission to quote his pathological report, to Dr. George Fujimoto for his cooperation with the infrared analyses, and to Dr. E. G. Holmstrom for his support and interest in this investigation.

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RIBONUCLEASE

VI PARTIAL PURIFICATION AND CHARACTERIZATION OF THE RIBONUCLEASES OF RAT LIVER MITOCHONDRIA*

By JAY S ROTH

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The problem of the mechanism of the synthesis of RNA¹ is of importance in obtaining an understanding of the fundamental chemistry involved in growth and cell division. The recent demonstration by Grunberg-Manago and coworkers (2, 3) that enzymes obtained from certain bacteria are able to synthesize and degrade RNA-like compounds has given added impetus to the study of enzymes related to RNA metabolism. One of these enzymes that has been intensively investigated is pancreatic RNase, and the properties, action, and structure of the crystalline enzyme are moderately well known (4-6). There is considerable evidence that RNases which may differ from pancreatic RNase exist in certain cells (7-10). In rat liver, the presence of two RNases has been demonstrated (11, 12), the one active maximally at pH 7.8 to 8.0 (alkaline RNase) appears to be similar to, or possibly identical with, crystalline pancreatic RNase, whereas the second, active maximally at pH 5.8 to 6.0 (acid RNase), is quite different in its properties. Since either, or both, of these enzymes may be normally involved in the synthesis or degradation of RNA in the mammalian cell, it is of considerable interest to isolate them and to study their properties, particularly in comparison to pancreatic RNase. This report describes a separation and partial purification of acid and alkaline RNases of rat liver mitochondria and some of the properties of the purified preparations.

Materials and Methods

Preparation of Mitochondria—Wistar strain rats weighing between 200 and 350 gm were used to provide the tissues. After etherization, the

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¹ The abbreviations used are RNA, ribonucleic acid, RNase, ribonuclease, ABC buffer, acetate, borate, cacodylate buffer, CMB, *p*-chloromercuribenzoate.

animals were exsanguinated and the livers removed and rinsed in ice-cold 0.25 M sucrose solution. Usually two livers were combined and homogenized in 9 volumes of 0.25 M sucrose solution with use of a close fitting Ten Broeck homogenizer. The total weight of liver used was generally between 10 and 14 gm. Mitochondria were prepared from the homogenate by standard techniques according to the method of Hogeboom *et al* (13), as modified by Schneider and Hogeboom (14), washed once with 0.25 M sucrose solution, and drained by inversion of the centrifuge tubes. The mitochondrial pellets were then made up to 100 ml. in glass-distilled water. These operations and all subsequent ones were carried out below 4°.

Enzyme Assays—These were carried out as previously described (15) with use of yeast RNA, uniformly labeled with P^{32} as substrate². Unless otherwise noted, the enzyme activities are expressed in terms of mg of acid-soluble phosphorus split per mg of N per hour. Nitrogen determinations were performed by the micro-Kjeldahl method. When ammonium sulfate was likely to interfere with N determination, the protein concentration was estimated by the procedure of Warburg and Christian (17) and also by a modified biuret reaction. Veronal-acetate buffers were prepared according to Michaelis (18), sodium chloride being omitted. ABC buffer was prepared by the method of de Duve *et al* (19), sodium deoxycholate solution was prepared fresh each week. Crystalline pancreatic RNase was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, and all concentrations of this enzyme were made up in 0.1 per cent gelatin solution.

Results

Effect of Buffers on Enzyme Activity, Selection of Buffer System—Because of several reports of the considerable influence of various salts and buffers on the activity of pancreatic RNase (20–22), it was deemed advisable to compare the action of several common buffers on RNase preparations in order to select the one most suitable for studies on both alkaline and acid RNase. Six different buffers were utilized and each was tested with

² It has been stated (16) that the assay method employed in this paper "involving measurement of the dialyzable P^{32} liberated is heavily dependent upon absolute uniformity of labeled PNA preparations made available." Actually, the method which measures the liberation of P^{32} soluble in 1 N HCl in 76 per cent ethanol does not involve dialysis. With a wide variety of labeled yeast preparations, differing in the extent of the labeling and slightly in chemical composition, it has been repeatedly demonstrated that assay of the same enzyme preparation with different substrates has resulted in values for activities differing by not more than the error of the method (± 5 per cent) when conversion of radioactivities to mg of P split per hour per mg of N has been made as previously described (15).

liver homogenate, separated mitochondria, and crystalline pancreatic RNase. The results are depicted in Table I. It is apparent that the choice of a buffer is extremely important, for a very large range of activity is obtained, depending on the buffer utilized. Edelhoch and Coleman (22) have shown that the activity of crystalline pancreatic RNase is greatly influenced by ionic strength, and it is possible that some of the differences in Table I are due to differences in ionic strength of the buffers used.

With alkaline RNase, phosphate is strongly inhibitory in the case of homogenate and mitochondria. With homogenate, the difference between

TABLE I

Effect of Buffers on Various Ribonuclease Preparations

The values are the percentages of maximal activity obtained with each preparation and are the average of three separate experiments

Preparation	PO ₄ ³⁻	ABC	Veronal-acetate	Acetate	Tris*	Citrate
Alkaline RNase buffer, pH 7.8, 0.2 M						
Rat liver homogenate	22.5	56.0	100	28.3	71.7	
" " mitochondria	44.3	46.0	66.7	60.1	100	
Crystalline pancreatic RNase	71.5	60.9	88.4	65.7	100	
Acid RNase buffer, pH 5.8, 0.1 M						
Rat liver homogenate	100	80.8	85.9	76.8		52.7
" " mitochondria	95.7	90.6	100	89.2		69.5
Crystalline pancreatic RNase	53.0	60.8	45.9	48.7		100

* Tris(hydroxymethyl)aminomethane

acetate and Veronal-acetate is particularly striking, the latter giving maximal activity and the former only 28.3 per cent of the maximum.

Although phosphate depresses, or gives poor activity with alkaline RNase, with acid RNase it gives the best response. Comparison of the effects of the same buffers on acid and alkaline RNases demonstrates the difference in response of these two enzymes and suggests that they are dissimilar. Citrate has a considerable activating effect on crystalline pancreatic RNase at pH 5.8 (and particularly in the pH range of 6.3 to 6.8)³ but is somewhat inhibitory to acid RNase in mitochondria and homogenate. The results of these experiments led to the selection of Veronal-acetate as the buffer of choice. With some exceptions, it gives the maximal activity with the tested preparations. Moreover, it covers the entire range of pH values likely to be of interest, and is easily prepared.

³ J. S. Roth, unpublished results.

Extraction and Purification of Acid and Alkaline RNases of Mitochondria—To 90 ml of separated mitochondria preparation in distilled water were added 14 ml of 1 per cent sodium deoxycholate solution. The mixture was stirred and then centrifuged at $8700 \times g$ (average) for 20 minutes in a Spinco model L ultracentrifuge. Most of the mitochondrial protein, including the RNases, is extracted by this procedure. After centrifugation, a small residue of granular material, overlying a waxy pellet, remains. The supernatant solution is slightly cloudy and straw-colored. Nearly complete recovery of mitochondrial RNase activity is obtained in this solution as may be seen by consideration of Table II.

The deoxycholate solution is next brought to 27.5 per cent saturation with ammonium sulfate by addition of solid salt. The mixture is stirred and then centrifuged at $8700 \times g$ for 20 minutes, and the precipitate is discarded. Ammonium sulfate is then added to 55 per cent saturation, and the resulting precipitate, which contains most of the RNase activity, is dissolved in a small quantity (usually 10 ml) of ice-cold water. Most of the precipitate dissolves, but a small residue of insoluble material remains. This residue is removed by centrifugation at $60,000 \times g$ for 20 minutes. The resulting solution is clear yellow to yellow-green in color. It is dialyzed against 1 liter of glass-distilled water in the cold for 24 hours with one change of water after 16 hours. The dialysate is discarded. A precipitate usually starts to form in the dialysis sac by the end of 10 hours. At the conclusion of the dialysis, the material in the dialysis sac (14 to 15 ml) is centrifuged at $60,000 \times g$ for 20 minutes. The precipitate contains the alkaline RNase and is purified as described below. The supernatant fluid which contains acid RNase, apparently quite free from alkaline RNase, may be used directly in studies of the properties of acid RNase or may be further purified as described below.

Purification of Alkaline RNase—The precipitate from the final centrifugation above is homogenized in a small glass homogenizer with about 5 ml of ice-cold 0.25 M sulfuric acid and allowed to stand overnight in the refrigerator. The resulting suspension is adjusted to pH 7.0 with NaOH and centrifuged at $60,000 \times g$ for 20 minutes. The supernatant fluid, which is a light straw color or colorless, is the final preparation of alkaline RNase.

Purification of Acid RNase—The supernatant solution prepared by centrifuging the material in the dialysis sac contains acid RNase and is referred to as acid RNase I. It is adjusted to pH 4.5 and heated at 50° for 5 minutes. A considerable amount of protein is coagulated by this treatment. The precipitate is centrifuged and discarded, and the remaining solution is treated with solid ammonium sulfate to 55 per cent saturation. The precipitate that forms is centrifuged at $60,000 \times g$ for

20 minutes, dialed, and then dissolved in a small quantity of ice-cold water. This final preparation is referred to as acid RNase II. The specific activities and recoveries of the purified RNases from mitochondria are given in Table II, from which it may be seen that the recovery of acid RNase is poor, amounting to only a small fraction of that originally present. The purification process is not too effective, and the final preparations of acid RNase II generally have from 3 to 5 times the specific activity of the original mitochondrial preparation. The acid RNase appears, however, to be relatively free from alkaline RNase⁴ as demonstrated by

TABLE II
*Specific Activities and Recoveries of Purified Ribonucleases from
Rat Liver Mitochondria*

Activity in mg of acid-alcohol-soluble P split per mg of N per hour

Fraction	Acid RNase			Alkaline RNase		
	Specific activity	N per ml	Recovery	Specific activity	N per ml	Recovery
		mg	per cent		mg	per cent
Homogenate	0.035	3.80		0.019	3.80	
Mitochondria	0.193	1.05	100	0.124	1.05	100
Deoxycholate-treated mitochondria	0.217	0.82	100	0.140	0.82	97.3
Supernatant from 27% (NH ₄) ₂ SO ₄ pptn	0.215	0.75*	90.2	0.137	0.75*	87.3
55% (NH ₄) ₂ SO ₄ ppt	0.175	3.34*	33.7	0.190	3.34*	54.0
0.25 M H ₂ SO ₄ -treated alkaline RNase				21.0	0.023	24.8
Acid RNase I	0.206	0.73*	12.2			
“ “ II	0.645	0.10*	5.2			

* Calculated from protein content determined by the biuret method. Other N determinations are by the micro-Kjeldahl method.

subsequent experiments on the effects of heating and pH on enzyme activity. The recovery of alkaline RNase is considerably better, amounting to approximately 25 per cent of that originally present in the mitochondria, and the purification is more effective. Very little nitrogen remains in the preparation of alkaline RNase and there does not appear to be any acid RNase activity present. Early attempts to separate acid and alkaline RNases by means of ammonium sulfate precipitation were not successful. In Table III are listed the results of an experiment in which the recoveries of acid and alkaline RNases were determined after precipitation from

⁴ Part of the difficulty in estimating the efficiency of the separation lies in the fact that alkaline RNase retains considerable activity when measured at pH 5.8 to 6.0, whereas acid RNase likewise retains a portion of its activity when assayed at pH 7.8 (see Fig. 1).

deoxycholate solution with various concentrations of ammonium sulfate. Consideration of the data in Table III indicates that the two enzymes respond somewhat differently to precipitation with ammonium sulfate, but at none of the concentrations employed was the separation promising.

TABLE III

Recovery of Ribonuclease Activity after Precipitation from Deoxycholate Solution by Ammonium Sulfate

Per cent saturation with $(\text{NH}_4)_2\text{SO}_4$	Per cent recovery of original activity	
	Acid RNase	Alkaline RNase
25	2.5	12.9
35	15.5	31.4
45	58.5	75.0
55	108.0	99.0
65	127.0	94.0

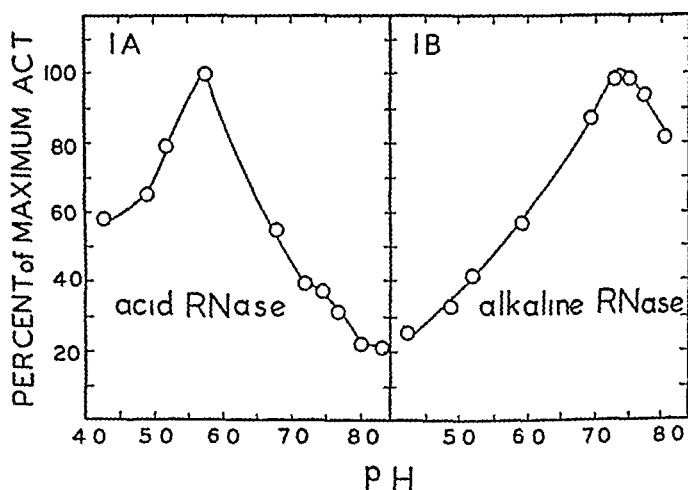


FIG 1 The effect of pH on the activity of purified preparations of acid (A) and alkaline (B) RNase of rat liver mitochondria. Veronal-acetate buffer. For experimental details consult the text.

It is possible, nevertheless, that salt precipitation under different pH conditions might be effective.

Effect of pH on Activities of Purified RNases—Aliquots of purified acid and alkaline RNases were assayed with use of Veronal-acetate buffers at pH values ranging from 4.2 to 8.4. Fig 1 illustrates the results of a typical experiment. Examination of the pH-activity curves indicates that the preparations of the enzymes are relatively free from the other RNase. The curve for alkaline RNase (Fig 1, B) shows no evidence at

all for the presence of the acid enzyme. In the curve for acid RNase (Fig 1, A) the small hump at pH 7.6 suggests that there may be some alkaline RNase present, but from the relative size of this hump, in comparison to that of the acid RNase peak, the amount of the alkaline RNase present is certainly no more than 5 per cent of the total activity. The curves in Fig 1 are of additional interest in that they allow an estimate to be made of the RNase activity of the acid and alkaline enzymes at the optimum pH of the other.⁴ Thus acid RNase at pH 7.8 retains about 30 per cent of its maximal activity measured at pH 6.0, whereas, at pH 5.8, alkaline RNase still exhibits about 50 per cent of its maximal activity.

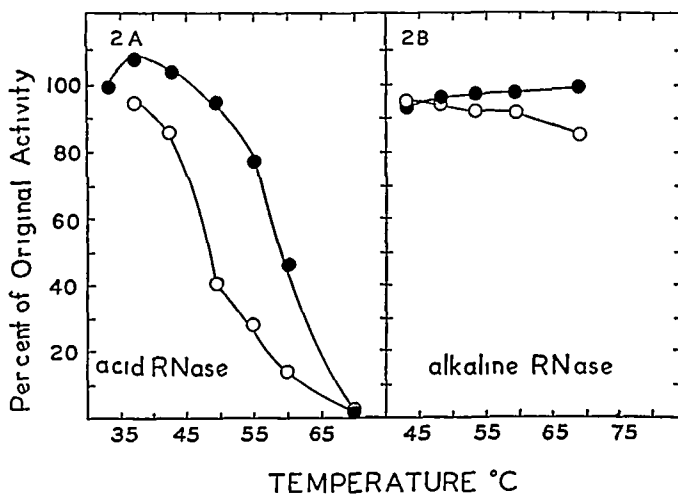


FIG 2 The heat stability of purified acid (A) and alkaline (B) RNase of rat liver mitochondria. Preparations heated at pH 5 (●) or 7.0 (○) for 5 minutes. For experimental details consult the text.

measured at pH 7.6. The optimal pH of purified rat liver mitochondrial alkaline RNase appears to be slightly lower than that of crystalline pancreatic RNase, and the activity of the former enzyme is greater than that of the latter when measured at pH 5.8. Whether these differences indicate a significant difference between rat liver alkaline RNase and crystalline pancreatic RNase cannot be assessed at the present time.

Heat Stability of Acid and Alkaline RNase—Purified preparations of acid and alkaline RNases were divided into two portions which were adjusted to pH 7.0 and 5.0, respectively, by the addition of small quantities of NaOH and HCl. Aliquots of the resulting solutions were heated for 5 minutes at various temperatures and then cooled in ice. The heated enzymes were then assayed by standard procedures and compared to an unheated control sample kept in ice. The results of a typical experiment are shown in Fig 2. The marked difference in heat stability of the two

enzymes is apparent. No detectable change was observed in the activity of alkaline RNase heated at pH 5.0, but there was a slight decline in activity when the enzyme was heated at pH 7.0. This behavior is similar to that of crystalline pancreatic RNase (23). Acid RNase, when heated at pH 5.0, was much more stable than when heated at pH 7.0. In both cases, however, activity was completely destroyed by heating for 5 minutes at 70°. The curves in Fig. 2 offer further evidence for the purity of the preparations. If alkaline RNase contained any significant amount of acid RNase, then heating at pH 5.0 and 70° should lead to a decrease in enzyme activity due to destruction of acid RNase. Similarly, if acid RNase contained significant amounts of alkaline RNase, then heating at pH 5.0 and 70° should leave a residue of activity due to stable alkaline RNase. The fact that neither of these effects was observed may be interpreted as indicating that each preparation is relatively free of the other enzyme.

Action of 0.25 M Sulfuric Acid on RNases and Cell Fractions—The Kunitz preparation of crystalline pancreatic RNase makes use of the extraction of tissue with ice-cold 0.25 N sulfuric acid for 24 hours or longer (24). Pirotte and Desreux (25) treated guinea pig liver homogenates with sulfuric acid and compared the activity of the fractions obtained subsequently with untreated fractions prepared in 0.88 M sucrose solution. In all cases, the untreated fraction (nuclei, mitochondria, microsomes, and supernatant) had only 5 to 14 per cent of the activity of the treated fraction. On the other hand, Rabinovitch (26) found no change in the activity of a pancreas homogenate treated with 0.15 N sulfuric acid. It appears that no careful study has been made of the effect of acid on acid and alkaline RNase activity of cell fractions or of purified RNase preparations. Since acid is used in the preparation of crystalline pancreatic RNase and in the purification procedure for alkaline RNase in this report, its effect on the RNase activity of various cell constituents is of interest.

A 1:10 rat liver homogenate in 0.25 M sucrose solution was prepared, and nuclear, mitochondrial, microsomal, and supernatant fractions were isolated. The nuclear fraction consisted simply of the material separated by centrifugation of the original homogenate at $650 \times g$ for 10 minutes, and undoubtedly contained some partially broken cells, as well as contaminating material from the other fractions. Aliquots of the various fractions were treated with 0.25 M sulfuric acid for 16 to 18 hours at 0°. At the end of this time the acid was neutralized with NaOH to pH 7.0, and the resulting suspensions were centrifuged at $10,000 \times g$ for 20 minutes. The clear supernatant solutions were assayed for acid and alkaline RNase activity. Two sets of controls consisted of identical aliquots treated with water or an equivalent quantity of sodium sulfate. All

solutions were made up to the same volume in preparation and analyzed for nitrogen content. Since there was little difference between the water-treated or sodium sulfate-treated fractions, results with the former have been omitted. A typical experiment is illustrated by the data in Tables IV and V. In Table IV are listed the total activities of the fractions, obtained by multiplying the activity per ml by the volume of each fraction. The following points are noteworthy. Acid treatment increases total alkaline RNase activity approximately 10-fold. The largest part of this increase arises from acid treatment of the supernatant fraction. It

TABLE IV
*Ribonuclease Activities of Rat Liver Fractions Treated with
0.25 M H_2SO_4 or 0.25 M Na_2SO_4*

Total activity in mg of acid-alcohol-soluble P split per hour

Fraction	Alkaline RNase*		Acid RNase†	
	Na_2SO_4 treated	H_2SO_4 treated	Na_2SO_4 treated	H_2SO_4 treated
Homogenate‡	16.7	214.3	89.0	153.7
Mitochondria	48.0	77.4	84.4	61.9
Microsomes	22.9	49.4	14.2	23.5
Supernatant	11.5	89.7	18.4	52.8
Nuclei	6.0	49.1	20.4	35.8
Total	88.4	265.6	137.4	174.0

* Assayed at pH 7.8

† Assayed at pH 5.9

‡ Prepared from 5.35 gm of rat liver

has been demonstrated (1) that large quantities of alkaline RNase are present in inactive form, possibly bound to a RNase inhibitor in this fraction. 0.25 M sulfonic acid destroys the inhibitor and releases the RNase activity. There is evidence, also, that nuclei may contain some inactive form of RNase as the increase in activity of the nuclear fraction is considerable. The alkaline RNase activities of mitochondria and microsomes are increased to a lesser, but significant, degree. The sum of the separate activities of the water- or sodium sulfate-treated fractions is considerably greater than the activity of the homogenate due to removal of inhibitor influence.

Treatment of homogenate with 0.25 M sulfonic acid increases acid RNase activity about 50 per cent. Acid treatment causes a loss in acid RNase activity of mitochondria but this is more than compensated for by in-

creases in the activities of the microsomal, nuclear, and supernatant fractions. The observed increase is probably not dependent on release of bound or inactive acid RNase in any fraction, but more likely is compounded from several effects. First, the large increase in alkaline RNase activity described above would affect acid RNase measurements since the alkaline enzyme still exhibits about 50 per cent of its activity at pH 5.8 to 6.0. Second, although RNase inhibitor is most effective in the pH range 6.8 to 8.0, it inhibits acid RNase to the extent of 30 to 40 per cent at pH 6.0 and its destruction by acid would result in a relative increase in acid RNase activity. Both of these effects would be partly offset, of

TABLE V
Specific Ribonuclease Activities of Rat Liver Fractions Treated with 0.25 M H_2SO_4 or Na_2SO_4

Specific activities in mg of acid-alcohol-soluble P split per mg of N per hour

Fraction	N content		Specific activity			
	Na ₂ SO ₄ treated	H ₂ SO ₄ treated	Alkaline RNase*		Acid RNase†	
			Na ₂ SO ₄ treated	H ₂ SO ₄ - treated	Na ₂ SO ₄ treated	H ₂ SO ₄ treated
	mg per ml	mg per ml				
Homogenate‡	3.07	0.38	0.01	1.08	0.06	0.78
Nuclei	1.41	0.18	0.03	1.09	0.06	0.80
Mitochondria	1.63	0.17	0.12	1.83	0.21	1.47
Microsomes	0.70	0.07	0.13	2.83	0.08	1.34
Supernatant	1.14	0.26	0.02	0.82	0.04	0.48

* Assayed at pH 7.8

† Assayed at pH 5.9

‡ Prepared from 5.35 gm of rat liver

course, by the destructive effect of sulfuric acid on acid RNase, since studies reported below with purified acid RNase indicate that at least 50 per cent of it is destroyed by the acid treatment.

In Table V are presented the specific activities and nitrogen contents of the various fractions. The specific activities of the fractions are greatly increased, in every instance, by acid treatment which, in addition to releasing, in some cases, additional RNase activity, also denatures and precipitates a large percentage of the protein of the fraction. Thus, this one-step procedure results in an increase of 50 to 100 times in the specific activity of alkaline RNase of homogenate.

Effect of 0.25 M H_2SO_4 on Purified Acid RNase and Crystalline Pancreatic RNase—Treatment of a purified preparation of acid RNase (acid RNase I) with 0.25 M sulfuric acid results in a decrease in acid RNase activity and

a corresponding increase in alkaline RNase activity. In a typical experiment, illustrated in Table VI, acid RNase and crystalline pancreatic RNase (0.05 mg per ml) were treated with 0.25 M sulfuric acid in the cold for 18 hours. Controls were treated with equivalent amounts of sodium sulfate. After neutralization to pH 7.0, the solutions were assayed at pH 5.9 and 7.8. Approximately 50 per cent of the activity in the acid RNase preparation was destroyed. However, since alkaline RNase activity increased correspondingly in this preparation, this loss in acid RNase activity probably represents more than a 50 per cent destruction of the latter enzyme, inasmuch as a part, if not all, of the remaining activity at pH 5.9 may be attributed to alkaline RNase. No significant change was noted in the activity of acid-treated crystalline pancreatic RNase at

TABLE VI

Effect of 0.25 M H₂SO₄ and 0.25 M Na₂SO₄ on Purified Acid Ribonuclease and Crystalline Pancreatic Ribonuclease

Relative activity in counts per minute

Substance	Na ₂ SO ₄ treated		H ₂ SO ₄ treated	
	Assayed at pH 5.8	Assayed at pH 7.8	Assayed at pH 5.8	Assayed at pH 7.8
Acid RNase I	1179	385	651	1056
" " I + CMB*	1434	1104		975
Crystalline pancreatic RNase	596	4441	550	4175

* 4×10^{-4} M *p*-chloromercuribenzoic acid

either pH. The observed increase in alkaline RNase activity could be explained in any of several ways. Sulfuric acid may effect a conversion of acid to alkaline RNase or there may be an inactive form of alkaline RNase present which is activated by acid. Further information on this question was obtained by treatment of the acid RNase preparation with 4×10^{-4} M CMB. When this was done there was an increase in alkaline RNase activity similar to that observed on sulfuric acid treatment. However, the activity of acid RNase was not affected. (The increase noted in Table VI may be accounted for by the increase in alkaline RNase activity.) These data suggest that the second possibility mentioned above may be the correct one, since CMB has been shown to release RNase activity from the supernatant fraction of rat liver (1). Unlike the supernatant fraction, however, the acid RNase preparation had no inhibitory activity on crystalline pancreatic RNase. It is of interest to note that Schneider and Hogeboom (27) reported a 50 per cent increase in RNase activity (measured at pH 5.0) of sonically disrupted mitochondria. De

Duve and coworkers (28) also studied the distribution and properties of RNase in cell fractions and found that the activity of acid RNase, which was located primarily in their heavy and light mitochondrial fraction, could be increased by repeated freezing and thawing, exposure to Triton X-100, or water, and incubation at 37°. In view of the finding in this report of latent RNase activity in a preparation obtained from mitochondria, the two studies are not in conflict. One question that remains to be answered is, does this latent or inactive RNase actually occur as an integral part of the mitochondrion or does it merely represent inactive RNase which has been absorbed onto the particulate from the supernatant fraction? Further experiments are necessary to answer this question.

DISCUSSION

The properties and distribution of the RNase system in the rat liver cell appear to be quite complicated, and at this time cannot be related to the physiological distribution of RNA. The presence of RNase in the cell nucleus remains in doubt. There is probably no acid RNase in this structure in rat liver (11, 27), but there is evidence for the occurrence of a small quantity of alkaline RNase. The possibility that an additional amount of inactive RNase occurs in the nucleus has been mentioned in this paper and also by others (25).

The mitochondria, although containing only a small fraction of the RNA of the cell, contain a large portion of the RNase activity. This activity is distributed between two enzymes differing markedly from one another. There may also be some latent RNase activity in mitochondria.

Microsomes exhibit considerable RNase activity but they may not inherently contain these enzymes to any great extent *in vivo*, since it has been shown that the small particulate fractions of the cell are capable of binding considerable quantities of RNase (27), probably by means of the ribonucleoprotein.

The soluble portion of the cell contains little RNase activity, but does contain a large amount of alkaline RNase in an inactive form, which may be released by treatment with strong acid or some sulfhydryl reagents (1). This inactive RNase in the supernatant fraction may be bound to a protein inhibitor which is not saturated with the enzyme, for it can inactivate added crystalline pancreatic RNase.

The presence of an RNase inhibitor in the supernatant fraction appears to be logical and necessary to prevent extensive degradation of ribonucleoprotein by the RNase of the mitochondria and possibly the microsomes. The RNase picture is further complicated by the existence of different enzymes in different tissues of the same animal. Thus, Kaplan and Hepfel (29) have isolated an enzyme from calf spleen which resembles pan-

creatic RNase in most of its properties but has a pH optimum of 6.5. Also, studies on the chromatographic behavior of pancreatic RNase, as well as the partial degradation by specific proteases, indicate that there may be several different forms of the enzyme all possessing unchanged enzymic activity (30-32).

In order to resolve some of the complicated questions, as yet little understood, particularly concerning the function and interactions of the RNase system, additional investigations on the enzymes in particulate fractions of liver and spleen are in progress. Further studies on the chromatographic behavior of acid and alkaline RNase of rat liver mitochondria, as well as their specificities and additional properties of the enzymes, will be reported later.

SUMMARY

1 Acid and alkaline ribonuclease (RNase) of rat liver mitochondria have been separated and partially purified by a process involving salt precipitation from deoxycholate solution, followed by dialysis and treatment with 0.25 M sulfuric acid.

2 Alkaline RNase resembles crystalline pancreatic RNase in its stability to heat and treatment with sulfuric acid, as well as in the effects of salts and buffers.

3 Acid RNase is heat-labile and unstable to treatment with 0.25 M sulfuric acid at 0° for 16 to 18 hours.

4 The effect of incubating rat liver homogenate and fractions with 0.25 M sulfuric acid at 0° for 16 to 18 hours has been studied. Large increases in the total and specific alkaline RNase activity of homogenate, as well as nuclear and supernatant fractions, were observed, and an explanation of these increases was discussed.

5 The action of 0.25 M sulfuric acid on purified preparations of acid and alkaline RNase was investigated.

6 The effect of various buffers on RNase activity has been reported.

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THE TOXIC ACTION OF OXYGEN ON GLUCOSE AND PYRUVATE OXIDATION IN HEART HOMOGENATES*

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The experiments reported in this paper are concerned with the inhibitory action of oxygen at 1 atmosphere pressure on the carbohydrate metabolism of heart muscle studied *in vitro*

It has been observed by several investigators that oxygen at 1 atmosphere pressure may have a harmful effect on the metabolism of mammalian tissue *in vitro* or on individual enzyme systems (1-5). Dickens (6, 7) and Stadie and coworkers (8, 9) have studied systematically the effect of oxygen at elevated tension on tissue respiration and on the activity of various enzymes. The conclusions arrived at by the two groups of investigators were similar. Many enzymes, particularly those dependent upon sulfhydryl groups for activity, are more or less easily inactivated by oxygen pressures of 1 atmosphere or greater and many are completely resistant to the toxic action of oxygen. The enzymes inactivated by oxygen *in vitro* usually require 1 or more hours of exposure to 1 atmosphere of oxygen before inhibition becomes apparent. Even at oxygen pressures as high as 8 atmospheres, tissue respiration *in vitro* is only slowly inhibited. It has also been a general finding that enzymes in tissue extracts or homogenates are more readily inactivated than the same enzymes when present in the intact cells of tissue slices. For these and other reasons, it has been difficult to relate the effects of oxygen observed *in vitro* to the severe symptoms of oxygen poisoning produced within 15 to 30 minutes in animals exposed to 3 to 8 atmospheres of oxygen (5, 10, 11). The review by Dickens (5) contains an excellent critical evaluation of the work that has been carried out on the toxic action of oxygen on enzymes and tissue metabolism. From recent work on the mechanism of oxygen poisoning in the intact animal, it appears that there is a close similarity between the toxic effects of excess radiation and of oxygen at high pressure (12, 13).

In the present work, the effect of 1 atmosphere of oxygen on the multi-enzyme systems involved in the oxidation of glucose and pyruvate has been studied. Rat heart homogenates fortified with KCl and diphos-

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phopyridine nucleotide (DPN) were used. Such homogenates have the ability to catalyze at a rapid rate the complete oxidation of glucose and pyruvate to carbon dioxide and water (14, 15). It was found that, compared to controls in air, the rate of oxidation of both glucose and pyruvate was consistently depressed by 1 atmosphere of oxygen and that the inhibitory effect of oxygen was profoundly influenced by metal ions in the reaction medium. A study of this toxic action of oxygen and its modification by metal ions is presented in this paper.

Methods

Enzyme System—Male albino rats, weighing 150 to 200 gm, were used and fed *ad libitum*. For each experiment an animal was killed by decapitation and the heart was placed in cold medium of the following composition: 0.040 M sodium phosphate, 0.095 M NaCl, pH 7.2. After being blotted on filter paper, the heart was cut into small pieces and ground by hand in a glass homogenizer in 3 ml of chilled medium of the same composition. The suspension was filtered through cheesecloth to remove gross particles. An additional 5 ml of chilled medium were added to the homogenate. In later experiments, the heart was weighed before homogenization and the total volume of medium added was 1.5 ml per 100 mg of wet weight. In these later experiments, we also substituted KCl for NaCl in the homogenization medium, with which the enzyme system is more stable. The homogenates may be cleared of large particles and cellular debris by centrifugation at about $200 \times g$ for 5 minutes with little or no loss of activity.

Incubation—The homogenates oxidized glucose and pyruvate vigorously when incubated at 37° in the presence of DPN and KCl or $MgCl_2$. The composition of the reaction system varied in different experiments as explained. In a typical experiment, the system consisted of the following: 0.5 ml of homogenate (corresponding to 30 to 40 mg of tissue), 0.05 ml of 5 per cent glucose (or 0.1 ml of 0.1 M sodium pyruvate), 0.1 ml of 3 per cent DPN, 0.5 ml of 0.3 M sorbitol, and 0.5 ml of 0.15 M KCl. The reaction mixtures were incubated at 37° in Warburg flasks containing alkali in the side compartments. In the absence of added substrate, there was considerable oxygen uptake for the first 20 to 40 minutes caused by the oxidation of substrates initially present in the homogenate. With added glucose or pyruvate, activity continued for 2 to 3 hours or more. Under optimal conditions, the oxygen uptake of the system with glucose as substrate corresponds to 400 to 450 μ moles per gm of wet tissue per hour. This is approximately 5 times the respiration of rat heart slices incubated *in vitro* with glucose as substrate. Utilization of glucose by the heart homogenate was determined by measuring the initial and final glucose

concentration in the medium by the method of Miller and Van Slyke (16). Activity was determined with air, 4 per cent oxygen (96 per cent nitrogen), 7.4 per cent oxygen (92.6 per cent nitrogen), or 100 per cent oxygen in the gas phase. CoCl_2 , CuSO_4 , MgCl_2 , or the chelating agent ethylenediamine-tetraacetic acid (EDTA or Veisene) was added in some of the experiments as indicated.

Chemicals—DPN was obtained from the Pabst Laboratories and solutions of it were neutralized with NaOH before use. Sodium pyruvate was prepared from pyruvic acid and crystallized.

EXPERIMENTAL

Inhibition by 100 Per Cent Oxygen—When homogenates were incubated with glucose or pyruvate as substrates at 37° , the rates of oxygen uptake were initially linear with time and the same in air and oxygen. However, in all experiments, the subsequent decline in the rates of reaction of the systems in oxygen began sooner and was more pronounced than in the controls in air (Table I). Typical experiments illustrating the effect of oxygen on the oxidation of glucose and pyruvate are recorded in Fig. 1.

Table I contains the results of a series of experiments in which oxidation of glucose by rat heart homogenate was studied with air or 100 per cent oxygen in the gas phase. Total oxygen uptake and utilization of glucose are recorded. In all of the experiments, glucose was present in excess so that at least one-half of the added glucose remained at the end of the period of incubation. The experiments in Table I show that both oxygen uptake and glucose utilization were depressed in 100 per cent oxygen compared to the controls in air.

It was shown in a previous paper (15) that glucose oxidation by heart homogenate is highly dependent upon the cations present in the reaction medium. When sodium ions are the only cations added, there is no activity. Addition of KCl, MgCl_2 , or both will activate the system. KCl was present in most of the experiments reported in Table I. However, we have also included some experiments in which MgCl_2 or both KCl and MgCl_2 were added. Inactivation by 100 per cent oxygen was found under all of these experimental conditions. The results (Table I) show that the oxygen uptake was approximately 6 times the utilization of glucose, indicating that most of the glucose used during the experiment was completely oxidized. The rate of reaction during the 70 to 100 minute period in per cent of the initial rate is recorded in the seventh column. It is seen that the activity of the system decreased considerably during incubation in air, but that in all experiments the decline of the rate of reaction was greater in oxygen than in air. The times at which the rates of oxygen uptake of the samples in oxygen had declined to 50 per cent of the rates of the con-

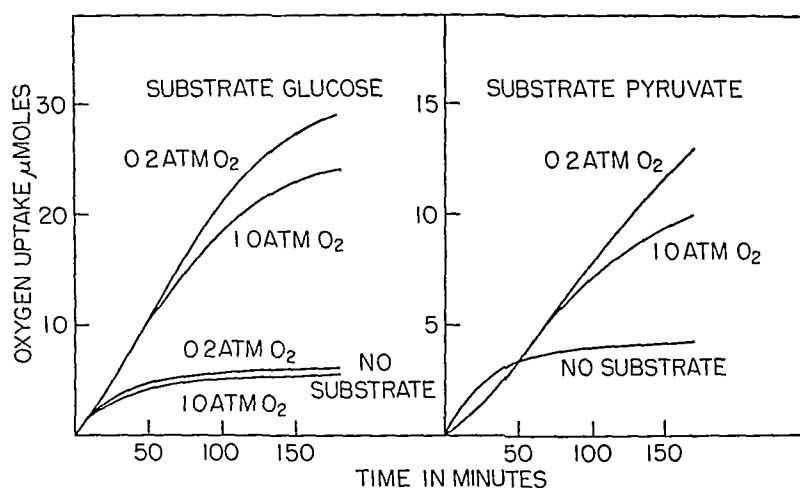


FIG 1 Oxidation of glucose and pyruvate by rat heart homogenate in air and oxygen The reaction system was as described under "Methods" Glucose when present, 0.008 M, sodium pyruvate when present, 0.0065 M ATM = atmosphere

TABLE I

Glucose Oxidation by Rat Heart Homogenate in Air and Oxygen

The reaction system had the following composition 0.028 M NaCl, 0.012 M sodium phosphate, 1.8 mg of DPN per ml, 0.008 M glucose, KCl, when added, 0.045 M, MgCl₂, when added, 0.0012 M, sorbitol added to constant osmolarity (0.270), pH 7.2, 40 to 50 mg of tissue Total volume, 1.70 ml Each figure is the mean of duplicate determinations

Experi- ment No	Activating ions	Gas phase	Time	Oxygen uptake	Glucose utilization	Oxygen uptake 70-100 min 10-40 min × 100	Time for 50 per cent inhibition*
			min	μmoles	μmoles	per cent	min
1	K ⁺	Air	120	17.5	2.9	66	85
		Oxygen	120	13.6	1.3	24	
	K ⁺	Air	120	17.4	1.8	42	70
		Oxygen	120	12.5	0.6	14	
3	K ⁺	Air	150	21.0	3.5	90	105
		Oxygen	150	15.8	3.0	69	
4	K ⁺	Air	210	25.1	3.8	82	115
		Oxygen	210	19.3	3.4	78	
5	Mg ⁺⁺	Air	120	22.3	3.8	39	75
		Oxygen	120	19.9	3.2	17	
6	K ⁺ , Mg ⁺⁺	Air	120	15.6	3.5	29	85
		Oxygen	120	12.6	1.8	17	
7	K ⁺ , Mg ⁺⁺	Air	120	28.7	5.7	45	80
		Oxygen	120	25.8	5.1	20	

* The time, *t*, at which the rate of oxygen uptake of the sample in oxygen, during the interval *t* ± 5 minutes, had decreased to 50 per cent of the rate of the control in air during the same interval of time

tiols in an are given in the eighth column of Table I. It is apparent that the time necessary to reach a certain degree of inactivation varies widely from experiment to experiment. Apparently, both the extent and the time of onset of oxygen toxicity vary from one tissue preparation to another.

Effect of Cupric Ions—The variation in the time of onset of oxygen poisoning of the system made it likely that some factors, so far unrecognized, influenced the toxic action of oxygen in these experiments. One possibility was that trace metals present in the tissue or introduced by the reagents affected the results. Cupric ions are known to catalyze the oxidation by oxygen of compounds containing sulfhydryl groups (17), ascorbic acid (18), and possibly other essential tissue components. The cupric ion is a normal tissue constituent in trace amounts. The oxidation of glucose by the rat heart homogenate was, therefore, studied in air and oxygen in the presence and absence of small amounts of CuSO_4 . It was shown in separate experiments that Na_2SO_4 in high concentrations (compared to the concentrations of CuSO_4 used) had no effect on the system, so that any effects of CuSO_4 could be assumed to be caused by the cupric ions. CuSO_4 in concentrations above 2×10^{-5} M was found to be highly toxic to the enzyme system when studied with air or with oxygen in the gas phase. At somewhat lower concentrations, the system in oxygen was affected to a much greater extent than was the control in air, and at concentrations of CuSO_4 in the range of 5×10^{-6} M to 10^{-5} M the copper ion had little or no effect on the enzyme system incubated in air but strongly inhibited the system incubated in oxygen. These findings are illustrated in Fig. 2. Similar effects of cupric ions were observed when pyruvate was the substrate.

The inhibitory action of the cupric ion could be abolished by the addition to the medium of the chelating agent EDTA (Table II).

These observations constitute indirect evidence for the hypothesis that the toxic effect of oxygen on metabolism is brought about by an oxidation of one or more essential metabolites or enzymes to an inactive form.

Dickens (6) demonstrated with brain slices and homogenates that certain cations, particularly Co^{++} and Mn^{++} , when present in small concentrations, protected against the toxic action of oxygen at increased tension. The effect of the addition to the medium of a small amount of CoCl_2 was studied in the presence and absence of added cupric ions. These experiments are reported in Table III.

In air, neither CuSO_4 nor CoCl_2 in the concentrations used had any effect on the enzyme system. Oxygen uptake and glucose utilization were significantly depressed in 100 per cent oxygen. Traces of cupric ions greatly increased the toxic effect of oxygen, and cobalt ions had no significant effect in the absence of added CuSO_4 , but did significantly diminish the

inhibition by oxygen in the presence of added CuSO_4 . The nature of this effect of cobalt ions may be similar to the action of cobalt and manganese

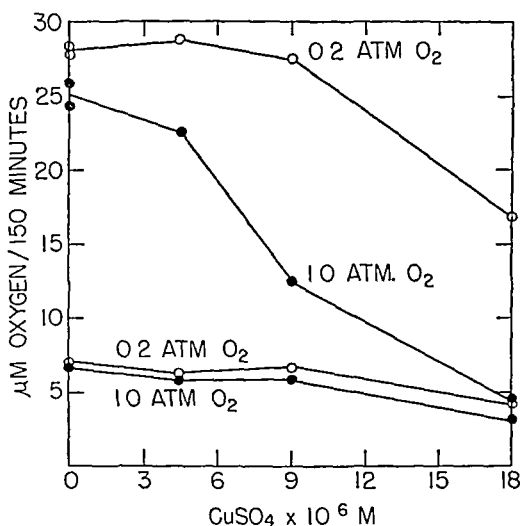


FIG 2 The effect of copper ions on glucose oxidation by rat heart homogenate in air and oxygen. The reaction system was as described under "Methods". The abscissa is the concentration of CuSO_4 and the ordinate is the total oxygen uptake during 150 minutes of incubation at 37° . Two upper curves were obtained with glucose as substrate, and the two lower curves without substrate added. ATM = atmosphere.

TABLE II

Glucose Oxidation by Rat Heart Homogenate, Effect of CuSO_4 and EDTA

The reaction system was as described under "Methods". CuSO_4 and EDTA were added as indicated below. Time of experiment, 130 minutes. Each figure is the mean of duplicate determinations.

Gas phase	CuSO_4	EDTA	Oxygen uptake	Glucose utilization
	<i>M</i>	<i>M</i>	μmoles	μmoles
Air	0	0	24.9	4.2
Oxygen	0	0	23.1	3.2
Air	1.5×10^{-5}	0	22.0	3.5
Oxygen	1.5×10^{-5}	0	13.3	1.7
Air	1.5×10^{-5}	6×10^{-5}	28.9	3.4
Oxygen	1.5×10^{-5}	6×10^{-5}	28.4	3.9

ions observed by Dickens (6, 7) and may consist of an antagonism to ions that accelerate oxygen poisoning. These ions may be naturally present in the tissue or, as in our experiments, they may be added to the reaction medium. It should be pointed out that the concentration of cupric ions

added in these experiments is actually lower than the concentration naturally present in human plasma (19)

TABLE III

Effect of Copper and Cobalt Ions on Oxidation of Glucose by Rat Heart Homogenate in Air and Oxygen

Five experiments were performed with separate heart homogenates. In addition to the regular components of the reaction mixture, described under "Methods," replicates contained (1) no extra added ions, (2) CuSO_4 , (3) CoCl_2 , (4) $\text{CuSO}_4 + \text{CoCl}_2$ as indicated below, gas phase was air or 100 per cent oxygen. In each experiment (120 minutes of incubation at 37°) the total oxygen uptake and glucose utilization were measured and the mean activity of the controls in air (and without CuSO_4 or CoCl_2) was designated 100 per cent. The activities of the other samples were expressed in per cent of the control. The figures below for each experimental category are the means of the per cent differences from the controls \pm the standard error of the mean. The figures in parentheses represent the number of individual determinations.

Category No	Ions added	Oxygen uptake		Glucose utilization	
		In air	In oxygen	In air	In oxygen
		per cent*	per cent*	per cent*	per cent*
I	None	0 (9)	-14 \pm 1.6 (9)	0 (9)	-14 \pm 3.3 (9)
II	9×10^{-6} M CuSO_4	0 \pm 2.3 (4)	-46 \pm 3.7 (9)	-4 \pm 6.1 (4)	-62 \pm 6.9 (9)
III	3.8×10^{-5} M CoCl_2	+1 \pm 1.7 (4)	-7 \pm 1.0 (7)	-8 \pm 4.1 (4)	-18 \pm 5.9 (7)
IV	9×10^{-6} M CuSO_4 + 3.8×10^{-5} M CoCl_2	0 \pm 1.1 (5)	-31 \pm 3.2 (8)	+4 \pm 3.2 (5)	-44 \pm 2.5 (8)
	Effect of cobalt in presence of copper (Categories IV-II)	0 \pm 2.5	+15 \pm 4.9 $t = 3.1$ $P < 0.01$	+8 \pm 6.9	+18 \pm 7.3 $t = 2.4$ $P < 0.01$

* Per cent differences from the activity of the control in air and in the absence of added copper and cobalt ions (see above)

Effect of EDTA—The observation that the cupric ion so strongly accentuates oxygen toxicity and that other ions also influence the phenomenon made it likely that chelating agents would modify oxygen toxicity. The effect of the chelating agent EDTA was accordingly studied. This substance combines strongly with the calcium ion, less strongly with magnesium, and also removes cupric ions from solution. When EDTA was added to a rat heart homogenate activated by KCl and with glucose as

substrate, it was found that at low concentrations (6×10^{-5} M or lower) EDTA had no effect or caused only a slight depression of the initial rate of reaction. If the concentration was increased above 10^{-4} M, however, the initial rate was definitely depressed. At a concentration of 10^{-3} M, EDTA caused complete inhibition. The inhibition appeared to be caused by the removal of magnesium ions from the reaction medium since the addition of an equivalent or greater amount of $MgCl_2$ restored activity completely. When an adequate concentration of KCl is present, glucose oxidation will proceed rapidly without added $MgCl_2$, but it is apparent that the magnesium ions present in the tissue itself are necessary for activity.

Although EDTA in small concentrations had little or no effect on the initial rate of reaction, it did influence the activity of the enzyme preparations after incubation for 1 hour or more at 37° . The general action of EDTA at a concentration of 6×10^{-5} M was that of stabilizing the system, *i e.*, of delaying the gradual fall in the rate of reaction that began at different times with every enzyme preparation studied. This stabilization of the system with EDTA occurred both in air and in oxygen but was significantly greater in oxygen than in air. In oxygen the time of the fall in activity (compared to the control in air) was, therefore, delayed and the difference in activity between the sample in air and oxygen was less pronounced. The observation that EDTA prolongs the activity of the system both in air and in oxygen makes it reasonable to assume that the fall in activity of the system in air, as in oxygen, is at least partly a result of the toxic action of oxygen, *i e.*, that oxygen in this cell-free enzyme system is toxic at the tension present in air. If this be true, the enzyme system should be more stable when incubated under tensions of oxygen lower than 0.2 atmosphere than it is in air or oxygen. The oxygen uptake of the heart muscle enzyme system was therefore determined in the presence of glucose with 4 per cent oxygen (96 per cent nitrogen), air, or 100 per cent oxygen in the gas phase. At each tension of oxygen the effect of the addition of 6×10^{-5} M EDTA was also determined (Experiment 1, Table IV). In two subsequent experiments (Experiments 2 and 3, Table IV) the lowest oxygen concentration was 7.4 rather than 4 per cent. It was realized that at a concentration of 4 per cent oxygen almost all of the oxygen present was used up at the end of the experiment and that, under such conditions, diffusion of oxygen into the solution might become a limiting factor in the measurement of oxygen uptake. With 7.4 per cent oxygen in the gas phase, the concentration of oxygen at the end of an experiment will still be about half of the initial concentration, even with the most active enzyme preparation studied.

For each experiment we have recorded the initial rate of reaction in micromoles of oxygen per hour calculated from the rate during the 30 to 50

minute interval of the experiment We chose this interval for calculation of the initial rate of reaction, since at 30 minutes the control without glucose

TABLE IV

Glucose Oxidation by Rat Heart Homogenate at Different Concentrations of Oxygen

Reaction system, Experiment 1 0.5 ml of homogenate (about 40 mg of tissue) in 0.040 M sodium phosphate, 0.095 M NaCl, pH 7.2 Final concentrations of components 0.012 M sodium phosphate, 0.029 M NaCl, 0.045 M KCl, 0.090 M sorbitol, 1.8 mg per ml of DPN, 0.0084 M glucose Total volume, 1.65 ml EDTA, when present, 6×10^{-5} M Experiments 2 and 3 0.5 ml of homogenate (31 mg of tissue) in 0.040 M sodium phosphate, 0.095 M KCl, pH 7.2 Final concentrations of components 0.017 M sodium phosphate, 0.074 M KCl, 0.065 M sorbitol, 2.6 mg per ml of DPN, 0.012 M glucose Total volume, 1.15 ml EDTA, when present, 9×10^{-5} M Oxygen uptake determined in Warburg vessels at 37°, CO₂ absorbed by alkali Gas phase, 4 per cent O₂-96 per cent N₂, 7.4 per cent O₂-92.6 per cent N₂, air, or 100 per cent O₂, as indicated below For each experiment the initial rate of oxygen uptake (calculated from the 30 to 50 minute rate) is recorded in micromoles per hour Average rates during subsequent 40 minute intervals are given in per cent of the initial rate Each figure is the mean of duplicate determinations

Experiment No	No EDTA	With EDTA	No EDTA	With EDTA	No EDTA	With EDTA
	4 per cent oxygen		Air		100 per cent oxygen	
1 Initial rate, μ moles per hr	10.4	8.9	11.4	8.9	10.5	9.0
" " %						
70-110 min	93	104	84	99	66	78
110-150 "	54	85	36	93	15	74
150-190 "	18	80	11	83	9	65
	7.4 per cent oxygen					
2 Initial rate, μ moles per hr	14.1	12.8	14.6	13.2	14.1	12.5
" " %						
70-110 min	79	87	81	88	67	81
110-150 "	76	84	67	82	56	76
150-190 "	54	64	29	69	11	55
190-230 "	46	51	10	59	11	49
230-270 "	38	42	5	51	7	42
3 Initial rate, μ moles per hr	13.4	11.4	13.5	11.6	14.1	12.5
" " %						
70-110 min	81	83	81	89	74	84
110-150 "	78	82	58	77	30	77
150-190 "	58	73	22	76	9	62
190-230 "	51	59	5	64	4	55
230-270 "	51	55	4	58	3	43

added had almost ceased to take up oxygen, and significant oxygen poisoning did not occur earlier than 50 minutes from the start of the experiment

It is seen that the initial rate of reaction was almost the same at the different tensions of oxygen, indicating that the respiratory enzymes are saturated with oxygen even at 4 per cent concentrations. EDTA, at a concentration of 6×10^{-5} M in Experiment 1 and 9×10^{-5} M in Experiments 2 and 3, depressed the initial oxygen uptake slightly. The mean oxygen uptake values during subsequent 40 minute periods of incubation were calculated and expressed in per cent of the initial rate. There was in all cases a progressive fall in activity with time. This fall started earlier and was more pronounced in oxygen than in air. It was also found that the oxygen uptake was maintained better in 4 or 7.4 per cent oxygen than in air. EDTA protected the enzyme system against inactivation at all concentrations of oxygen. This protective effect of EDTA was particularly pronounced with 100 per cent oxygen in the gas phase. With EDTA present, the enzyme system has a remarkable stability. Even after 250 minutes of incubation at 37° , activity was still at a level of 50 per cent of the initial rate (Experiments 2 and 3).

DISCUSSION

In the experiments reported here the toxic action of oxygen on a cell-free multienzyme system is well demonstrated. The inhibitory action of oxygen is manifested by 100 per cent oxygen and also at the concentration of oxygen present in air. However, there is considerable evidence that most enzymes, when present in the intact cells of tissue slices or in the animal, are less susceptible to oxygen poisoning than they are when present in cell-free preparations such as the heart homogenate used in these experiments. This may be an indication that protective mechanisms are present in the cell that counteract the oxidizing potential of molecular oxygen. It has been observed that susceptibility to poisoning by oxygen varies widely from species to species (4, 20) and with the physiological state of the animal (21). It is possible that such differences in resistance to oxygen toxicity may parallel changes in the protective mechanisms of the cell. As an extreme case, one may consider the strictly anaerobic bacterium as an organism without any protective mechanism against oxygen poisoning.

That copper ions greatly accentuate oxygen poisoning and that the chelating agent EDTA exerts a protective action strongly support the view that the toxic action of oxygen on tissue metabolism is caused by a metal-catalyzed oxidation of one or more easily oxidizable tissue constituents. There is much evidence that the tissue constituents involved may be enzymes or coenzymes containing sulfhydryl groups. (1) The so called SH—enzymes are especially vulnerable to inactivation by oxygen. (2) The SH—coenzymes, glutathione and coenzyme A, have been shown by Barron (22) to be oxidized in water solution by high pressures of oxygen. (3)

Copper ions, found in our experiments to accentuate oxygen toxicity, are known to catalyze the oxidation of SH— groups (17)

The importance that the *in vitro* studies of oxygen toxicity may have for our understanding of the phenomenon of oxygen poisoning in the intact animal remains to be decided. The difficulties involved in conciliating the results from studies of tissue preparations with observations made on intact animals have been discussed by Stadie and Haugaard (11) and by Dickens (5). It should be pointed out, however, that recent studies with intact animals by Geischman *et al* (23) support the hypothesis that oxygen poisoning is caused by an oxidation of essential SH— groups. These authors found that mice were protected against death from high oxygen pressure by previous injection of the sulfhydryl compounds glutathione, cysteine, β -mercaptoethylamine, or BAL. Of importance in this connection are also the studies of Lambertsen *et al* (24), who estimated that the oxygen tension in the brain cells of man exposed to toxic pressures of oxygen (3 to 4 atmospheres) was far below the tension of oxygen in the inspired gas. These considerations would tend to indicate that the tensions of oxygen that are toxic to cells *in vivo* are of the same order of magnitude as the oxygen tensions found to be toxic to tissue metabolism *in vitro*.

Much remains to be done before we understand the mechanism of oxygen poisoning. However, the evidence that has accumulated in favor of the hypothesis that oxidation of essential SH— groups is involved is impressive.

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SUMMARY

The toxic effect of oxygen on the enzyme systems in heart muscle oxidizing glucose and pyruvate has been studied. It was found that oxygen at a pressure of 1 atmosphere (compared to air) produced a gradual inhibition of enzyme activity. Cupric ions in trace amounts greatly accentuated this toxic action of oxygen. The chelating agent, ethylenediaminetetraacetic acid, protected against inactivation of the enzyme systems by oxygen. The relation of these findings to the phenomenon of oxygen poisoning has been discussed.

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STUDIES ON THE EFFECT OF VITAMIN B₆ ON 5-HYDROXY-TRYPTAMINE (SEROTONIN) FORMATION

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It is becoming apparent that 5-hydroxytryptamine (serotonin) may be an important humoral agent (1-3), and information concerning its metabolism may be of value in elucidating its physiological roles. An important catalyst involved in the biosynthesis of serotonin is the enzyme 5-hydroxytryptophan (5HTP) decarboxylase (4). As a result of the actions of this enzyme, administered 5HTP is converted to serotonin in many tissues, including brain (5). Many of the pharmacological effects of this amino acid were shown to result from its ability to penetrate into the central nervous system, where it is converted to serotonin by 5HTP decarboxylase in brain.

It was previously reported that 5-hydroxytryptophan decarboxylase is inhibited by carbonyl reagents, suggesting that the coenzyme in the conversion of 5HTP to serotonin was pyridoxal phosphate (4). However, with purified preparations of guinea pig kidney, it was not possible to show more than 30 per cent stimulation upon addition of pyridoxal phosphate. Similar findings were later reported by Beiler and Martin (6). Subsequently, Buxton and Sinclair (7) found that in vitamin B₆-deficient rats the activity of kidney 5HTP decarboxylase was much lower than in normal rats and that the activity could be restored upon addition of pyridoxal phosphate *in vitro*.

The studies reported here represent additional evidence that pyridoxal phosphate is the coenzyme of 5HTP decarboxylase. The effects of vitamin B₆ deficiency and semicarbazide on serotonin production *in vivo* have also been investigated.

Materials and Methods

Materials—Pyridoxal phosphate (65 per cent purity) was generously donated by Dr. E. A. Peterson of the National Cancer Institute. Sero-

* Part of the work presented here will be submitted by Herbert Weissbach in a thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry, George Washington University, Washington, D. C.

tonin, 5HTP, and semicarbazide were obtained from commercial sources. Chickens were used in these studies because experiments on vitamin B₆ deficiency in this species were in progress for other purposes. Vitamin B₆-deficient chickens were prepared by being placed on artificial diets at the time of hatching. One group, made acutely deficient by a diet completely devoid of vitamin B₆, exhibited symptoms of pyridoxine deficiency after 9 to 12 days. The other group received a diet containing approximately 10 per cent of the minimal vitamin B₆ requirement, and showed symptoms after 4 to 5 weeks. Controls for this group consisted of animals limited to the same food intake containing optimal amounts of vitamin B₆.

Methods—Serotonin in tissues was determined by a method previously described (8). Whole blood was assayed for serotonin upon precipitation of proteins¹ with ZnSO₄ and NaOH, as previously described (9). 5-Hydroxytryptophan decarboxylase was assayed by measuring serotonin formation from 5HTP according to the procedure of Clark *et al* (4). *o*-Tyramine was assayed colorimetrically after extraction with *n*-butanol, as described by Mitoma *et al* (10).

EXPERIMENTAL

Dissociation of 5HTP Decarboxylase from Its Coenzyme—Although rat kidney is a relatively poor source of 5HTP decarboxylase, it was found that the enzyme in this tissue could be dissociated from pyridoxal phosphate more readily than in other tissues which contain more enzyme, *i.e.* guinea pig and hog kidney. The following procedure was used to make preparations requiring pyridoxal phosphate for decarboxylation of 5HTP. Rat kidneys were removed, immediately frozen, and stored at -10° for several days². After the kidneys had thawed, purification of 5HTP decarboxylase was carried out in a room at a constant temperature of $3-5^{\circ}$. The tissue was homogenized in 4 times the volume of distilled water and centrifuged for 30 minutes in a Servall centrifuge at high speed. After removal of the fatty layer the supernatant extract was dialyzed against distilled water overnight.

The dialyzed preparation at this stage was found to be relatively inactive unless pyridoxal phosphate was added. Pyridoxal phosphate produced little stimulation (Table I) unless the tissue was first subjected to freezing and thawing. This process apparently had little effect on the apoenzyme. Similar freezing and thawing techniques did not, however, affect the pyridoxal phosphate requirement of guinea pig kidney decarboxylase.

¹ Chicken blood contains sufficient amounts of serotonin (2 to 3 γ per ml) to assay directly in deproteinized blood.

² The usual procedure was to store the frozen kidneys over the weekend. Details of the time factors in the freezing and thawing were not investigated.

Effect of Semicarbazide on 5HTP Decarboxylase Activity in Vitro—It was shown previously that semicarbazide at concentrations of 10^{-2} M inhibited 5HTP decarboxylase almost completely and that this inhibition could not be reversed to any great extent by the addition of as much as 200 γ of pyridoxal phosphate (4). On the other hand, inhibition of 3,4-dihydroxyphenylalanine (dopa) decarboxylase by 10^{-2} M semicarbazide was almost completely reversed with 200 γ of pyridoxal phosphate, showing a distinct difference between these two enzymes. If semicarbazide inhibits because of interaction with pyridoxal phosphate, then excess pyridoxal phosphate should reverse the inhibition. Further studies with smaller amounts of semicarbazide have shown that the inhibition can be

TABLE I
Dissociation of Rat Kidney 5HTP Decarboxylase by Freezing and Thawing

Pyridoxal phosphate added	Control tissue*	Tissue after freezing and thawing*
γ		
0	1 00	0 28
1	0 95	0 68
2	1 31	
4	1 19	0 94
8	1 10	1 16

* The values represent micromoles of serotonin formed per gm of kidney per hour

Each flask contained 1.0 ml of enzyme, 10 μ moles of DL-5HTP, 0.3 ml of 1 M phosphate buffer, pH 8.1, 0.1 ml of octyl alcohol, and water to make a total volume of 3.5 ml. Incubation was carried out on a Dubnoff metabolic shaker at 37° for 30 minutes.

reversed with pyridoxal phosphate (Table II). The difference between dopa and 5HTP decarboxylase, previously reported, is difficult to explain by means of interaction of semicarbazide with the coenzyme alone.

Effect of Vitamin B₆ Deficiency on Tissue Serotonin Levels and 5HTP Decarboxylase Activity—The data presented in Table III indicate that in vitamin B₆-deficient animals the levels of serotonin in the tissues are markedly diminished. It would have been desirable to compare the 5HTP decarboxylase activity of tissues from vitamin B₆-deficient chicks with those of normal chicks and to demonstrate a requirement of pyridoxal phosphate in the deficient animals. However, 5HTP decarboxylase activity in even normal chicken tissues, including slices, was found to be so low as to make this type of experiment impossible. 5HTP was, however, readily converted to serotonin when administered *in vivo*. It was possible, therefore, to show that after a given dose of 5HTP less serotonin appeared in the tissues of vitamin B₆-deficient animals than in controls (Table IV). It is

apparent that less serotonin was present in the tissues of the vitamin B₆-deficient animals to begin with, and that upon administration of 5HTP the net increase in serotonin was much less

TABLE II
Inhibition of 5HTP Decarboxylase with Semicarbazide and Its Reversal with Pyridoxal Phosphate

Semicarbazide <i>μmoles</i>	Serotonin formed	
	Without pyridoxal phosphate	With pyridoxal phosphate
0	121	165
0.67	38	120
2.7	10	90
13.3	8.4	36
26.6	5.2	27

The enzyme used in these studies was a 10,000 × *g* supernatant solution of guinea pig kidney. An amount equivalent to 100 mg of original tissue was preincubated for 5 minutes with the indicated amounts of semicarbazide and with 0.5 μ mole of pyridoxal phosphate, after which 2 μ moles of DL-5HTP were added. The other details of the incubation were similar to those shown in Table I.

TABLE III
Serotonin Levels in Normal and Vitamin B₆-Deficient Chicks

Tissue	Serotonin	
	Control	Vitamin B ₆ deficient
	<i>γ per gm</i>	<i>γ per gm</i>
Brain	1.1 (± 0.3)	0.4 (± 0.06)
Intestine	5.3 (± 1.2)	1.2 (± 0.4)
Liver	1.6*	0.4*
Blood	2.9*	0.5 (± 0.08)

* Tissues from four animals were pooled and analyzed.

The chicks used in this experiment were those made chronically deficient and their pair-fed controls. Unless otherwise stated, each value represents the average obtained from six animals. The values in parentheses represent the mean deviation.

Effect of Semicarbazide in Vivo—The ability of semicarbazide to interact with pyridoxal phosphate *in vitro*, and thereby to inactivate 5HTP decarboxylase, suggested that administration of this carbonyl reagent might produce a serotonin deficiency similar to that observed in vitamin B₆-deficient animals.

Administration of semicarbazide to guinea pigs in amounts sufficient to

result in death in 1 to 2 hours (250 mg per kilo) did not produce a detectable decrease in the level of serotonin in the brain. However, a period of 1 to 2 hours may have been short compared to the normal rate of serotonin synthesis.

TABLE IV
Serotonin Levels in Normal and Vitamin B₆-Deficient Chicks after Administration of 5HTP

Tissue	Serotonin		
	Control	After 5HTP	Δ
	γ per gm	γ per gm	γ per gm
Normal liver	1.6	10.8	9.2
Vitamin B ₆ -deficient liver	0.4	2.0	1.6
Normal brain	0.8	5.0	4.2
Vitamin B ₆ -deficient brain	0.5	3.2	2.7

Each figure represents the pooled tissues of three animals. The values presented are typical of those observed in two experiments. The animals received 240 mg per kilo of DL-5HTP and were killed 2 hours later.

TABLE V
Serotonin Levels in Normal and Semicarbazide-Treated Mice after Administration of 5HTP

Experiment	Tissue serotonin*	Increase
	γ per gm	
Control	2.4	
Semicarbazide	2.2	
5HTP	8.8	6.4
" + semicarbazide	6.5	4.3

* Homogenates of whole mice were assayed.

Semicarbazide (240 mg per kilo) was administered 30 minutes before 5HTP (200 mg per kilo), the animals were killed 1 hour later. Each figure represents the average obtained in two separate experiments. In each experiment the values represent the pooled tissues of two animals.

The effect of semicarbazide on the amount of serotonin appearing in tissues after a given dose of 5HTP was also investigated. This was carried out in mice by pretreating them, before administering 5HTP, with the maximal amount of semicarbazide which would permit survival for a long enough time to carry out the experiment. They were killed 1 hour after receiving the 5HTP, and each animal was homogenized and assayed for serotonin. The results of this experiment (Table V) indicate that semi-

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THE METABOLISM OF MUCOPOLYSACCHARIDES IN ANIMALS

IV THE INFLUENCE OF INSULIN*

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The turnover of the acid mucopolysaccharides, hyaluronic acid (HA) and chondroitinsulfuric acid (CSA), of skin has been studied in normal rats (1) and in animals treated with the adrenocortical hormones, 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone (2). It was shown that the administration of these adrenal steroids to otherwise normal rats resulted in a decreased turnover of the mucopolysaccharides of skin.

The possibility that insulin participates in the metabolism of acid mucopolysaccharides was suggested by the finding that both the uronic acid and hexosamine moieties of HA derive from glucose (3-5). Since insulin regulates the utilization of glucose, it seemed reasonable to postulate a role for insulin in the biosynthesis of the mucopolysaccharides. It is the purpose of this paper to present evidence that mucopolysaccharide metabolism is impaired in the skin of alloxan-diabetic rats and is restored toward normal by insulin administration. A portion of this investigation was published previously in preliminary form (6).

Methods and Materials

Animals—Adult male rats of the Sprague-Dawley strain were used throughout this study. Except for animals on a limited food intake, the rats were fed Rockland chow and water *ad libitum*. Alloxan diabetes was induced by a single subcutaneous injection of 150 mg of alloxan monohydrate per kilo of body weight. Animals that did not lose weight were eliminated. 3 weeks after the administration of alloxan, blood glucose, determined on rats selected at random, ranged from 410 to 592 mg per 100 ml.

Insulin (NPH, Lilly) was injected subcutaneously in daily doses of 20 or 40 units per kilo of body weight. This dose was varied, because continued administration of the higher dose resulted in evidences of hypoglycemia in some animals. Glucose levels of 25 to 45 mg per 100 ml of blood were

* This investigation was aided by grants from the National Heart Institute (H-311) of the United States Public Health Service, the Herman Gordon Foundation, and the Variety Club of Illinois.

attained. On any day that the concentration fell below 25 mg per cent, as happened occasionally, the insulin injection was omitted.

Methods—Blood glucose was determined by the method of Nelson (7). C^{14} -carboxyl-labeled sodium acetate was prepared as described by Calvin *et al* (8). Uniformly labeled glucose- C^{14} was purchased from the Nuclear Instrument and Chemical Corporation, Chicago. The HA and CSA fractions were isolated from the skin as described in an earlier publication (9).

Aliquots of the separated mucopolysaccharides were oxidized to CO_2 and the C^{14} was counted as $BaCO_3$ in an internal gas flow counter, corrected to infinite thickness. A silver wire used in the combustion tube filling assured the complete removal of the S^{35} during the oxidation of the CSA fractions. The S^{35} concentration of the CSA fractions was determined as previously described (1).

The HA and CSA pool sizes in the skin of normal and diabetic rats were estimated by the method of isotope dilution. C^{14} -labeled HA and CSA were prepared *in vivo* by injecting rats with acetate-1- C^{14} and were isolated from the skin as described (9). The specific activity of the two mucopolysaccharides was determined and known quantities of each were added at the beginning of the extraction procedure to NaOH suspensions of defatted ground skins from normal and diabetic animals. After isolation of the mixture of labeled and unlabeled mucopolysaccharides, the specific activities of the HA and CSA fractions were determined and the amounts of mucopolysaccharides in the original skins were calculated (10). The values for the pool sizes of HA and CSA are expressed as mg per 100 gm of acetone-defatted dry skin.

EXPERIMENTAL

60 rats were divided into three experimental groups of equal size. One group of animals was used 3 weeks after the administration of alloxan. A second group of untreated animals served as controls, while a third group of normal animals was maintained on half the average daily food intake for 3 weeks prior to and during the experiment. The weight loss of the latter group was similar to that of the diabetic animals throughout the experimental period. Each of the 60 rats was injected once subcutaneously with 80 μc of acetate-1- C^{14} and 2.7 μc of $Na_2S^{35}O_4$ as an isotonic mixture. Ten rats in each group were sacrificed 1 and 5 days after the injection.

The uptake of C^{14} by HA and of C^{14} and S^{35} by CSA was strikingly less for diabetic than that for normal rats (Fig. 1). No such decrease was evident in the fasted rats. Since the weight lost by the animals on a restricted food intake duplicated that lost by the diabetic animals, weight

loss *per se* appears to have no influence on the uptake of C^{14} and S^{35} by the mucopolysaccharides of skin. The average body weights of the three groups of animals at the time of isotope administration were 355, 212, and 210 gm for the normal, fasted, and diabetic rats, respectively.

The disappearance of HA- C^{14} , CSA- C^{14} , and CSA- S^{35} 4 days after maximal labeling is illustrated in Fig 1. The C^{14} concentration of the CSA fraction from the diabetic animals has been omitted, since the radioactivity of the $BaCO_3$ from this sample was too low for accurate counting. While

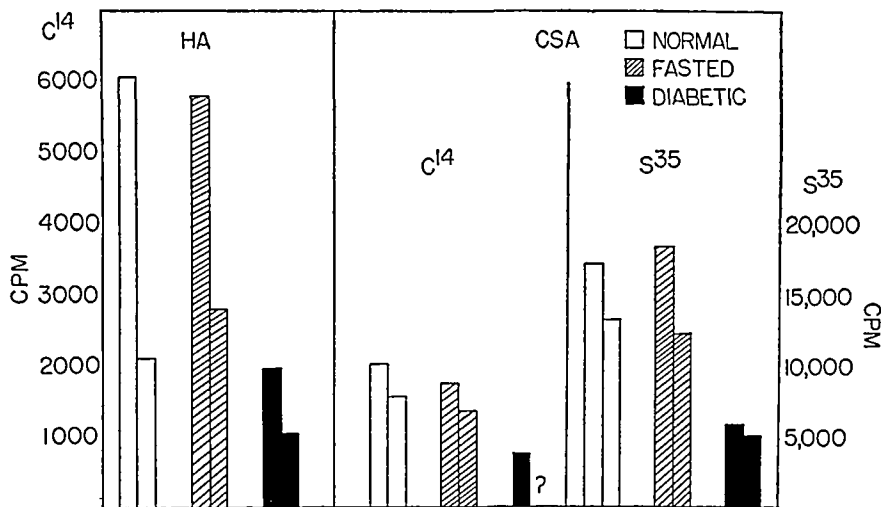


FIG 1 A comparison of the C^{14} concentration of HA and of the C^{14} and S^{35} concentrations of CSA among the three experimental groups following the administration of acetate-1- C^{14} and $Na_2S^{35}O_4$. The left and right bars of each pair represent the values 1 and 5 days, respectively, after the injection of the isotopes. The value of the CSA- C^{14} at 5 days is omitted in the case of the diabetic rats. See the text for an explanation.

half life times calculated from two points are not accurate, the values serve to indicate gross changes in turnover. The apparent half life times of 2.6 days for HA and 11.0 and 10.8 days for CSA (based on the C^{14} and S^{35} , respectively) found in the skin of normal animals agree with those obtained previously from more detailed decay curves (1, 2). In the diabetic animal, however, the turnover is considerably slower, as evidenced by an apparent half life of 4.5 days for HA and 20.9 days for CSA (based on S^{35}).

The results of this experiment indicated a decreased capacity to metabolize acid mucopolysaccharides in diabetic animals. A fall in concentration of these substances might therefore be anticipated. Since methods for isolating the mucopolysaccharides are not quantitative, attempts were

made to estimate possible changes in mucopolysaccharide concentrations by utilizing the method of isotope dilution. The results, indicated in Table I, demonstrate a marked decrease in HA concentration and a less striking decrease in CSA concentration. These determinations of pool size permitted the calculation of the turnover rates presented in Table I. The marked difference between diabetic and normal animals is evident. The lower turnover rates previously reported (6) were calculated from the quantities of HA and CSA isolated from skin by methods which are not quantitative.

The sizes of the HA and CSA pools in the skin of the fasted animals were not measured, hence turnover rates comparable to those calculated for the normal and diabetic rats could not be obtained.

TABLE I

Comparison of Pool Size and Turnover Rate in Normal and Diabetic Rats

	Condition	Pool size*	Turnover rate†
		mg per 100 gm	mg per 100 gm per day
HA	Normal	215	58
	Diabetic	88	14
CSA	Normal	187	12
	Diabetic	145	5

* Pool size as determined by the isotope dilution method and expressed as mg per 100 gm of acetone-defatted dry skin

† Turnover rate = (pool size)/($t_{1/2} \times 1.44$) (11) ($t_{1/2}$ = half life time)

The use of acetate-1- C^{14} effects specific labeling of the *N*-acetyl component of the mucopolysaccharide molecules (12). Since acetate utilization is decreased in the diabetic animal (13, 14), it may be argued that the observed results reflect alterations in acetate metabolism. Although the similarity of the C^{14} and S^{35} data would appear to invalidate this objection, another experiment was undertaken in which glucose-U- C^{14} as well as $Na_2S^{35}O_4$ was utilized. In the same experiment the effects of insulin on both normal and diabetic rats were studied. Four experimental groups were used. At appropriate times, as designated below, each animal received a single subcutaneous injection of an isotonic mixture containing 6.7 μ c of glucose-U- C^{14} and 13.3 μ c of $Na_2S^{35}O_4$. Two groups were made diabetic as described above. 3 weeks later one-half of the diabetic animals was injected with the radioactive mixture. The remainder was treated daily with 20 or 40 units of insulin per kilo of body weight for 1 week before the administration of the radioactive material and daily thereafter until sacrifice. The two other groups of animals consisted of non-

diabetic rats One group served as a normal control, while the other was injected daily with 20 or 40 units of insulin per kilo of body weight before and after receiving the isotopes in a manner identical to that used in the treatment of the diabetic animal

Eight to ten rats in each group were sacrificed at intervals of 1, 5, and 17 days after the administration of radioactive material and the HA and CSA fractions were isolated from the respective pools of skin

A comparison of the left bars of the diabetic and normal groups (Fig 2) indicates a marked decrease in incorporation of isotope in both HA and CSA in the diabetic animal This finding is entirely in accord with the re-

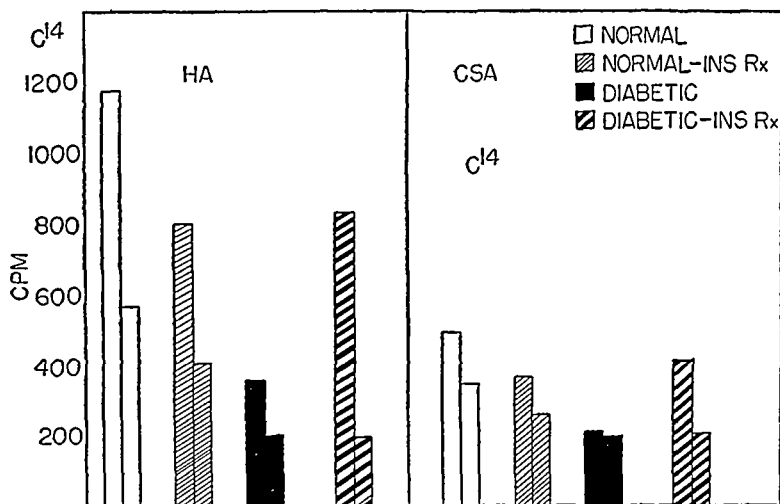


FIG 2 A comparison of the C^{14} concentration of HA and of CSA of the various groups 1 (left bar) and 5 (right bar) days after the administration of glucose- $U-C^{14}$ and $Na_2S^{35}O_4$

sults obtained when acetate- $1-C^{14}$ was employed as a precursor Calculation of half life times for HA again showed some prolongation in diabetic animals (5.0 days compared with 3.8 days for normal), although these differences were not as striking as those observed for CSA or those obtained in the previous experiment

The sulfate data (Fig 3) demonstrate, as in the previous experiment, a marked inhibition of isotope uptake in diabetic animals, although the differences in decay (half life times) are not as evident

The administration of insulin to diabetic animals restores the defect of uptake toward normal as illustrated by the data for C^{14} in HA (Fig 2) and for both C^{14} and S^{35} in CSA (Figs 2 and 3) The half life times were actually shorter than normal in this group (HA- C^{14} , 1.9 days, CSA- C^{14} , 3.9 days, and CSA- S^{35} , 6.3 days) This is not surprising, since these ani-

mals were hypoglycemic. The effect of insulin in normal animals was somewhat variable and not as striking as in the diabetic animals, probably because the diabetic animal is more sensitive to insulin.

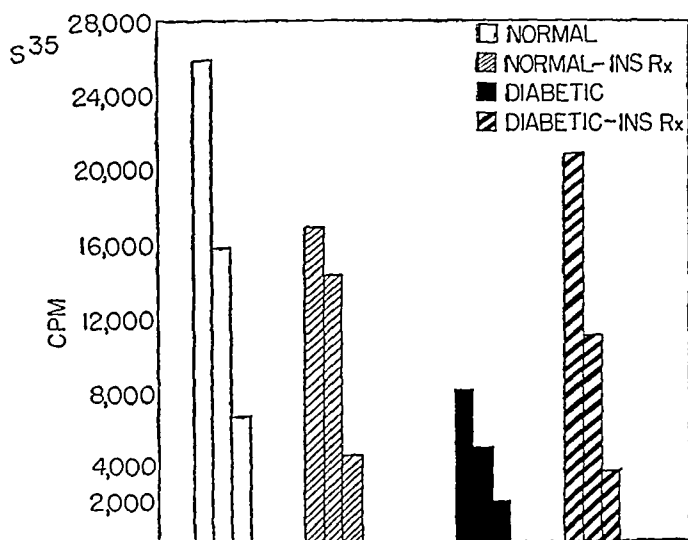


FIG 3 A comparison of the S^{35} concentration of CSA of the various groups following the administration of glucose- $U-C^{14}$ and $Na_2S^{35}O_4$. The bars in each group, read from left to right, represent values 1, 5, and 17 days after the injection of the isotopes.

DISCUSSION

In the present study an attempt was made to relate the pancreas to the synthesis of acid mucopolysaccharides. The data show that in diabetic rats the uptake of C^{14} by HA and of C^{14} and S^{35} by CSA is diminished, the sizes of the mucopolysaccharide pools in the skin are decreased, and the HA and CSA turnover rates are slower than normal. In the insulin-treated diabetic animal, the metabolism of the skin mucopolysaccharides assumes a more normal character.

The radioactivity at zero time of the HA and CSA fractions isolated from the skin of the diabetic animals was approximately one-third that of the same fractions isolated from the skin of the normal animals when either acetate- $1-C^{14}$ or glucose- $U-C^{14}$ and $Na_2S^{35}O_4$ were administered. These substances are precursors of specific moieties of the HA and CSA molecules (1, 12). This fact, taken together with the finding that the uptake of the isotopes was reduced to the same extent with each precursor used, confirms and supports the conclusion published previously (1) that the various components of the respective acid mucopolysaccharides turn over at the same rate. Furthermore, the observations argue against the

view that an increased initial dilution due to larger than normal body pools of acetate and glucose is responsible for the results in the diabetic animal, for it is unlikely that the acetate, glucose, and sulfate pools were increased to exactly the same extent

The results of this investigation indicate that the synthesis of the connective tissue mucopolysaccharides is inhibited in the insulin-deficient animal. That the decreased turnover of these substances in the diabetic rat results from a defect in glucose utilization seems likely. Such a concept would also account for the decreased turnover of the mucopolysaccharides in the skin of rats injected with adrenal cortical hormones (2).

Irrespective of its mechanism of action, a role of insulin in the metabolism of the acid mucopolysaccharides may have considerable biological implications. The retarded wound healing, the increased susceptibility to infection, and the accelerated vascular degeneration characteristic of diabetes mellitus may reflect a decreased ability to synthesize acid mucopolysaccharides.

SUMMARY

1 The turnover of the mucopolysaccharides, hyaluronic acid (HA) and chondroitinsulfuric acid (CSA), was estimated in the skin of diabetic, fasted, insulin-treated non-diabetic and insulin-treated diabetic rats, following the administration of the precursors, acetate-1-C¹⁴ or glucose-U-C¹⁴ and Na₂S³⁵O₄.

2 The uptake of C¹⁴ by HA and CSA and of S³⁵ by CSA isolated from the skin of diabetic animals is approximately one-third that found in the normal animals. Insulin treatment restored the values toward normal.

3 It is suggested that insulin participates in the metabolism of acid mucopolysaccharides.

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THE INDUCTION OF TRYPTOPHAN PEROXIDASE IN THE ISOLATED PERFUSED LIVER*

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Tryptophan peroxidase (TP), an inducible hepatic enzyme (1, 2), responds in the intact rabbit or rat to the specific substrate L-tryptophan by a rapid increase in activity. Smaller increase in tryptophan peroxidase activity may be mediated through the adrenal gland by a variety of non-substrate compounds (3), hypophyseal growth hormone and other hormonal agents may also affect the level of tryptophan peroxidase activity (3, 4). In an effort to eliminate all such variables except substrate concentration, we have used isolated perfused liver of rabbit and rat to study the induction of tryptophan peroxidase. The studies demonstrated TP induction in these organs.

EXPERIMENTAL

Livers were obtained from non-fasted male white New Zealand rabbits weighing about 2 kilos. Blood for perfusion was obtained from animals of the same strain weighing 4 to 5 kilos. In the rat liver perfusions, 200 gm male albino animals of the Sherman strain served as liver donors, and blood was obtained from larger animals (400 gm) of the same strain. The technique of Young, Prudden, and Stirman (5) was used to perfuse the rabbit livers. Isotope and chemical studies have shown that protein synthesis occurs in this perfusion system (6). The same procedure modified by a scaling down of the apparatus to approximately one-tenth of its original size was employed for perfusion of the rat livers. The perfusate consisted of a mixture of 50 to 60 per cent whole heparinized blood, a casein hydrolysate (5) which raised the amino acid nitrogen to 24 mg per cent, and L-tryptophan to a level of approximately 11.8 μ moles per ml of perfusate. Specific test compounds were added as desired.

Tryptophan peroxidase activity was assayed by the method of Knox and Mehler (7). Aromatic amines were determined by diazotization (8).

All data presented are from experiments characterized by (1) continuous

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† Fellow of the National Cancer Institute, September, 1954, to March, 1956.

bile production, (2) perfusion at a low head of perfusate pressure, (3) good gross appearance of the liver at the end of the perfusion, and (4) production of large amounts of acyl conjugates of the tryptophan metabolites

RESULTS AND DISCUSSION

Tryptophan peroxidase activity of liver samples is shown in Table I. All post-perfusion values are markedly higher than initial values, and the mean increase of 3.6 times was found for rabbit liver. Since the initial levels in the rabbit liver are lower than those found in the rat, it seemed desirable to determine whether increases in enzymatic activity could also be produced by perfusion of the isolated rat liver. As with rabbit liver, increases in peroxidase activity occurred, the activity being tripled in two perfusions of 2.5 and 4 hours duration.

TABLE I

Tryptophan Peroxidase Activity before and after Perfusion with L-Tryptophan

Species	No. of perfusions	Length of perfusion	Activity*		Increase
			Initial	Final	
		hrs			per cent
Rabbit	10	6	0.37	1.33	360
Rat	1	2.5	4.6	13.6	300
"	1	4	4.6	15.0	330

* Values are expressed in micromoles of kynurenine formed per gm of wet weight of liver per hour. No overlap in the initial and final figures was found.

A perfusion was performed on a rabbit liver and a rat liver, low levels (0.1 μ mole per ml) of tryptophan being employed. In both cases no change in tryptophan peroxidase activity was observed, the values after perfusion being identical with those observed before perfusion, 0.38 and 4.6 μ moles of kynurenine formed per hour per gm of wet weight of tissue for the rabbit and rat, respectively.

The results show that induction of TP takes place in isolated perfused liver¹. The magnitude of the response is only a third or a fourth of that usually seen in the intact animal but is about the same as that noted by Geschwind and Li for adrenalectomized rats (4).

Production of aryl amines by the liver was followed by hourly sampling of the perfusate. Fig. 1 shows the amount of aryl amines produced each hour plotted against time. The rate of aryl amine production increases as perfusion proceeds up to 4 hours, after which it decreases. Preliminary

¹ The induction of hepatic threonine dehydrase in perfused liver has recently been reported (9).

experiments in which a change to fresh perfusate was employed after 4 hours suggest that the accumulation of tryptophan metabolites in the perfusate may suppress tryptophan peroxidase induction, since aryl amine production will continue to increase upon the addition of fresh perfusate.

Pretreatment of rabbits with the methionine analogue, ethionine, or the addition of ethionine to the perfusate (Fig 1) containing the usual amount of tryptophan, greatly diminished aryl amine production. The addition of methionine to the perfusate containing the ethionine overcame the inhibition produced by the ethionine.

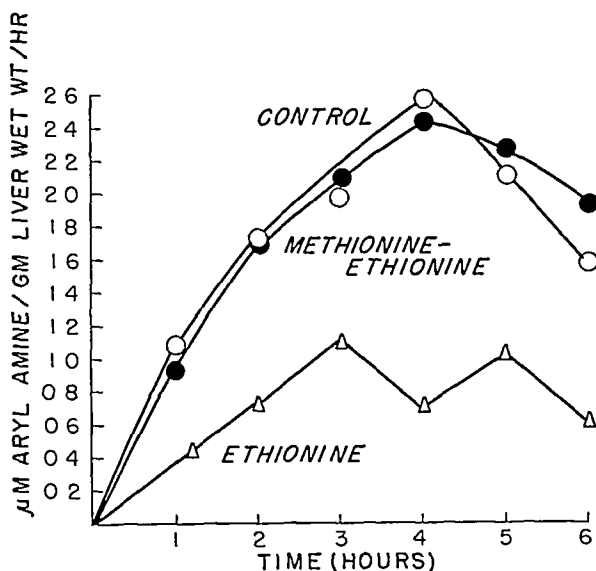


FIG 1 Aryl amine production in the perfused rabbit liver. L-Ethionine and L-methionine were added to the perfusate at a level of 18 μ moles per ml and 15 μ moles per ml, respectively.

The finding that ethionine inhibits the induction of tryptophan peroxidase in perfused liver is in accord with the *in vivo* observations of Lee and Williams (10), and can be taken as further evidence for the net synthesis of enzyme during the induction of tryptophan peroxidase.

The authors wish to express their appreciation to Dr John F. Prudden for use of his laboratory and for his help and suggestions throughout this work.

SUMMARY

Induction of hepatic tryptophan peroxidase takes place in the isolated perfused rabbit or rat liver, as demonstrated by assays of tryptophan

peroxidase activity of the liver before and after perfusion and by following the rate of aryl amine formation in the perfused liver. This enzymatic induction can be inhibited by ethionine and the inhibition in turn reversed by methionine.

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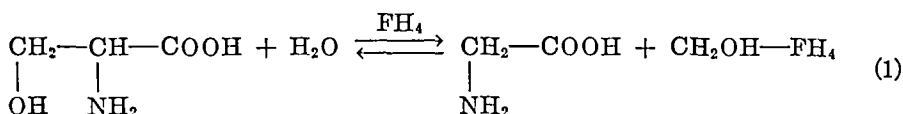
HYDROXYMETHYL TETRAHYDROFOLIC DEHYDROGENASE*

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The preceding paper of this series (2) described the conversion by the enzyme, *serine hydroxymethylase*, of L-serine to glycine and a coenzyme-bound 1-carbon fragment at the oxidation level of formaldehyde (*cf* Equation 1)



where FH_4 represents tetrahydrofolic acid¹ and $\text{CH}_2\text{OH—FH}_4$ represents the formaldehyde-coenzyme complex. Evidence was adduced also for the existence of a corollary enzyme which oxidizes the $\text{CH}_2\text{OH—FH}_4$ complex according to Equation 2



where CHO—FH_4 represents the formate-coenzyme complex. The existence of this enzyme, *hydroxymethyl tetrahydrofolic dehydrogenase*, in pigeon liver and beef liver extracts was demonstrated first by Jaenicke and Greenberg *et al* (3–5). The present paper describes the spectrophotometric assay, partial purification, and properties of this dehydrogenase.

* This work has been supported by a grant from the Life Insurance Medical Research Fund. A preliminary report was presented at the Forty-seventh meeting of the American Society of Biological Chemists at Atlantic City, April, 1956 (1).

Paper II in the series, "Folic acid coenzymes and active one-carbon units." For Paper I, see Huennekens *et al* (2).

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¹ The following abbreviations will be used: FH_4 , 5,6,7,8-tetrahydrofolic acid, $f^5\text{FH}_4$, N^5 -formyl tetrahydrofolic acid (folinic acid), $f^{10}\text{FH}_4$, N^{10} -formyl tetrahydrofolic acid, $f^{5,10}\text{FH}_4$, N^5 - N^{10} -methenyl derivative of FH_4 (anhydroleucovorin), hFH_4 , hydroxymethyl tetrahydrofolic acid (positions of the $\text{—CH}_2\text{OH}$ group on FH_4 not specified), TPN, TPNH, DPN, DPNH, oxidized and reduced tri- and diphosphopyridine nucleotides, PyP, pyridoxal phosphate, ATP, adenosine triphosphate, PCMB, *p*-chloromercuribenzoate, PCMPS, *p*-chloromercuriphenylsulfonic acid, GSH, GSSG, reduced and oxidized glutathione.

EXPERIMENTAL

Materials—GSSG was obtained from the Schwarz Laboratories, Inc, PCMB and PCMPs from the Sigma Chemical Company, and folic acid from the California Foundation for Biochemical Research. Calcium phosphate gel was prepared by the method of Keilin and Hartree (6). $f^6\text{FH}_4$ and $f^5\text{}^{10}\text{FH}_4$ were generously provided by Dr. E. L. R. Stokstad of the Lederle Laboratories Division, American Cyanamid Company. $f^5\text{}^{10}\text{FH}_4$ was converted quantitatively to $f^{10}\text{FH}_4$ by treatment with dilute base in the presence of 2-mercaptoethanol to prevent oxidation of the product (7). All other chemicals were obtained from the sources mentioned in Paper I of this series (2).

FH_4 was prepared by the catalytic hydrogenation of folic acid suspended in glacial acetic acid (8), essentially according to the method of O'Dell *et al.* (9). After hydrogenation was complete, the solution was transferred to a dry box in an atmosphere of N_2 and filtered directly into a flask which was immersed in a mixture of dry ice and acetone. The flask contents were then lyophilized to yield a white, fluffy preparation of FH_4 .

Preparations—Beef liver acetone powder extracts and Dowex-treated extracts were prepared as described previously (2). Extracts of pigeon liver acetone powder were prepared in an identical manner, except that high speed centrifugation ($10,000 \times g$ for 20 minutes) was necessary to obtain clear solutions. Unless otherwise specified, *beef liver* preparations were used in all experiments reported in this paper.

Partial purification of the hFH_4 dehydrogenase was carried out as follows: 100 ml of beef liver extract were adjusted to pH 6.2 with 1 N acetic acid, and the precipitate, obtained after centrifugation at $2300 \times g$ (International refrigerated centrifuge, model PR-1) for 10 minutes, was discarded. The supernatant fluid was treated with 20 ml of calcium phosphate gel (30 mg per ml), the pH being maintained at 6.2, and, after gentle mechanical stirring for 5 minutes, the gel was recovered by centrifugation. After the gel was washed three times by suspension and centrifugation with 50 ml of water, the enzyme was eluted successively with 30 and 20 ml of 0.1 M phosphate buffer, pH 7.5.

The activity of the partially purified enzyme declined rapidly during storage at $0-5^\circ$, or at -20° , in contrast to the original extract which was stable over a period of several weeks when stored in the frozen state. Addition of glycerol (33 per cent v/v) to the purified preparation largely prevents the deterioration upon storage.

TPNH oxidase² was isolated from brewers' yeast by the method of Green *et al.* (10).

² Unpublished results from this laboratory have shown that the flavoprotein isolated by Green *et al.* (10) is a TPNH oxidase (or diaphorase), which requires methylene blue as an electron carrier.

Methods—The manometric assay system was identical to that described previously (2), except for the omission of ATP and the addition of TPN (0.6 μ mole) and FH_4 (0.65 μ mole). The spectrophotometric assay system for TPN reduction (Equation 2 in the forward direction) consisted of the following components: 10 μ moles of L-serine (or 5 μ moles of HCHO), 0.6 μ mole of DL- FH_4 ,³ 0.6 μ mole of TPN, 100 μ moles of sodium phosphate buffer, pH 7.5, 0.2 ml of extract or 0.05 ml of partially purified enzyme, and water to make 3.0 ml. For assay of the DPN-linked HCHO dehydrogenase (11), the cuvettes contained 5 μ moles of HCHO, 0.75 μ mole of DPN, 10 μ moles of GSH, 100 μ moles of phosphate buffer, pH 7.5, 0.2 ml of extract, and water to make 3.0 ml. Blanks were identical except for the omission of substrate. For measuring the reaction represented by Equation 2 in reverse, the following components were used: 1 μ mole of DL- $f^{10}\text{FH}_4$, 0.55 μ mole of TPNH, 150 μ moles of phosphate buffer, pH 7.5, 10 μ moles of 2-mercaptoethanol, 0.2 ml of enzyme, and water to make 3.0 ml. The blank was identical except for the omission of $f^{10}\text{FH}_4$.

Spectrophotometric experiments were carried out at 340 $m\mu$ in 1 cm Corex cells in the Beckman DU spectrophotometer. The extinction coefficient for TPNH or DPNH at 340 $m\mu$ was taken as 6.22×10^6 sq cm per mole (12).

$f^{10}\text{FH}_4$ was estimated by conversion to $f^6\text{FH}_4$ upon acidification and was measured spectrophotometrically at 350 $m\mu$, with an extinction coefficient of 22.0×10^6 sq cm per mole (13).

RESULTS AND DISCUSSION

Reduction of TPN with Serine—As shown in Fig. 1, the enzyme preparation was able to catalyze the reduction of TPN in the presence of L-serine. Under the same conditions, glycine failed to reduce TPN. This indicated that the 1-carbon fragment, derived from the β -carbon of serine (*cf* Equation 1), was the actual substrate for the oxidation shown in Equation 2. These results are consistent with our previous data (2).

In Fig. 1, evidence is presented also that the increase in light absorption at 340 $m\mu$ is due actually to the production of TPNH. After the reaction had been allowed to proceed nearly to completion, a purified TPNH oxidase preparation, isolated from yeast, and methylene blue were added at the time indicated by the arrow, and the subsequent decline in light absorption was noted. The same experiment was repeated, except that GSSG was added at the arrow and the rapid disappearance of TPNH was again observed, due to the action of endogenous glutathione reductase (*cf* Equation 3).



³ FH_4 , prepared chemically by the hydrogenation of folic acid, is a DL mixture, owing to the asymmetric carbon atom at the 6 position.

Reduction of TPN with Formaldehyde—Recently, Kishuk (14) and Jaenicke (5) have shown that HCHO and FH_4 react together to form hFH_4 (cf Equation 4)



which serves as the cosubstrate with glycine for serine biosynthesis, *ie* Equation 1 in reverse. It was anticipated, therefore, that HCHO , in the presence of added FH_4 , would replace serine as a substrate for the TPN-

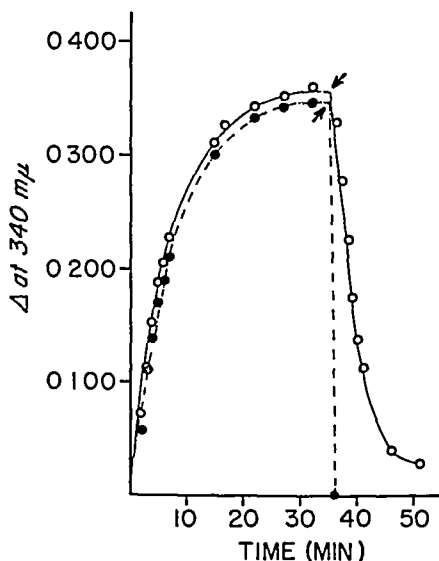


FIG 1 TPN reduction with serine. Standard spectrophotometric assay system, except that FH_4 was omitted and the enzyme was not pretreated with Dowex 1. The following additions were made to two identical experiments at the time indicated by arrows: O, 0.4 ml of TPNH oxidase and 0.1 ml of 0.01 per cent methylene blue, ●, 5 μ moles of GSSG.

linked hFH_4 dehydrogenase. In a typical experiment, in which the protocol for Fig 1 was used, 0.25 and 0.13 μ moles of TPNH were produced in 20 minutes when serine and HCHO , respectively, were used as substrates. The activity with HCHO as a substrate is somewhat lower than that with serine, indicating either that excess HCHO inhibits the dehydrogenase reaction or that the combination of HCHO and FH_4 is the rate-limiting step.

With serine as a substrate, TPN cannot be replaced by DPN. A more complex situation obtains, however, when HCHO is used as a substrate, since, in addition to the TPN-linked hFH_4 dehydrogenase, the crude enzyme preparation contains the DPN-linked, GSH-requiring HCHO dehydrogenase, described previously by Strittmatter and Ball (11). Treat-

ment of the crude extract with calcium phosphate gel (see "Experimental") largely removes the DPN-linked enzyme

In Dowex-treated enzyme preparations, the reduction of TPN with either serine or HCHO as substrate is absolutely dependent upon added FH_4 (see below). Also, as discussed below, the addition of graded amounts of PCMPS to the system produces essentially the same degree of inhibition of TPN reduction whether serine or HCHO is used as substrate. Thus, although serine and HCHO are each one step removed from the dehydrogenase reaction, it seems reasonable to assume that each gives

TABLE I
Requirement for PyP and FH_4

Experiment No	Additions	TPNH	O_2 uptake
		$\Delta \log I_0/I$	
I	None	0.268	7.6
	PyP	0.449	14.0
II	None	0.016	2.4
	FH_4	0.720	18.2

Standard assay systems. Experiment I, enzyme pretreated with semicarbazide (see Huennekens *et al.* (2) for the details), and 0.04 μmole of PyP was added as indicated. Duration of spectrophotometric experiment, 60 minutes, manometric experiment, 600 minutes. Experiment II, enzyme pretreated with Dowex 1, and 0.65 μmole of FH_4 was added as indicated. Duration of spectrophotometric experiment, 30 minutes, manometric experiment, 250 minutes.

rise to the common intermediate, hFH_4 (via reactions of Equations 1 and 4), which is the actual substrate for the dehydrogenase reaction (Equation 2). Until hFH_4 becomes readily available in purified form, serine or HCHO remains as the only convenient source of the actual substrate for the hFH_4 dehydrogenase.

Cofactor Requirements for Coupled System.—The requirement for pyridoxal phosphate in the serine hydroxymethylase reaction (Equation 1), previously demonstrated by the manometric assay, could be shown as well in the spectrophotometric system wherein the hydroxymethylase again is coupled with the dehydrogenase (*cf.* Table I). As before (2), the enzyme was pretreated by dialysis against semicarbazide to remove, or inactivate, the bound PyP. Approximately the same percentage stimulation with added PyP is observed in both the manometric and spectrophotometric test systems.

In a similar manner, the requirement for FH_4 , the other cofactor in

Equation 1, is demonstrated by parallel manometric and spectrophotometric assays (*cf* Table I). The dependence of reaction velocity upon concentration of FH_4 in the spectrophotometric system is illustrated in Fig 2. From a Lineweaver-Burk double reciprocal plot of these data (see the inset in Fig 2), the Michaelis constant (K_m) for DL- FH_4 is calculated to be $2.7 \times 10^{-5} \text{ M}$. The value for the active isomer³ would be $1.4 \times 10^{-5} \text{ M}$. It should be noted that the K_m for FH_4 in our system is of the

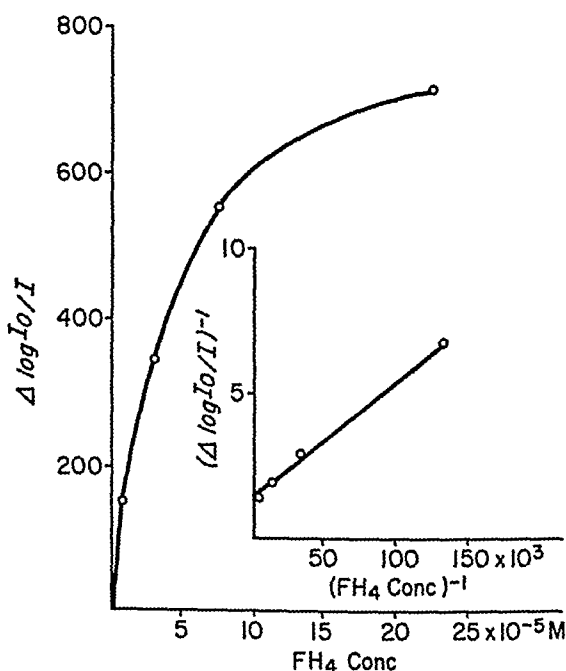


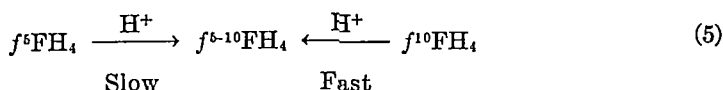
FIG 2 Effect of FH_4 concentration. Standard assay system, with a Dowex-treated enzyme and varying amounts of DL- FH_4 , as indicated. The optical density change ($\Delta \log I_0/I$) at $340 \text{ m}\mu$ was taken over the first 10 minute period after addition of enzyme. In the absence of added FH_4 , the blank had a value of 0.005. Inset, double reciprocal plot of FH_4 concentration (abscissa) versus $\Delta \log I_0/I$ (ordinate).

same order of magnitude as that calculated from the results reported by Alexander and Greenberg (15) for the serine hydroxymethylase from sheep liver ($K_m \cong 6.7 \times 10^{-5} \text{ M}$). The K_m value for FH_4 in Equation 4 has not been determined, since it is not yet known whether the reaction is chemical,⁴ enzymatic, or a combination of both. The absolute requirement for FH_4 in Equation 4 can be demonstrated, however, by experiments analogous to those in Table I. With HCHO as substrate for the coupled reactions of Equations 2 and 4, changes in optical density of 0.289 and

⁴ It has been shown that HCHO and FH_4 interact rapidly to form a complex spectroscopically similar to hFH_4 prepared enzymatically by a reversal of Equation 2 (M. J. Osborn, P. T. Talbert, and F. M. Huennekens, unpublished observations).

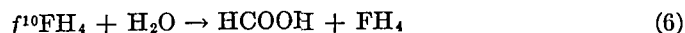
0.009 were obtained in 20 minutes in the presence and the absence of added FH_4 .

Product of Dehydrogenase Reaction—The product of the hFH_4 dehydrogenase reaction, written as $\text{CHO}-\text{FH}_4$ in Equation 2, was identified previously (3-5) and confirmed in this investigation as the N^{10} -formyl isomer of FH_4 . At the conclusion of a typical reaction (see the protocol for Fig. 1), the solutions in the blank and experimental cuvettes were deproteinized by treatment with cold perchloric acid to a final concentration of 3 per cent. With the protein-free filtrates, the absorption spectrum of the experimental sample was determined relative to the blank. A single absorption band was observed, centered at $350 \text{ m}\mu$. It should be noted that the original absorption of TPNH at $340 \text{ m}\mu$ is completely destroyed upon acidification. Furthermore, the addition of GSSG to the reaction mixture before acidification discharges the TPNH via the glutathione reductase reaction (*cf* Equation 3) and results in a solution having essentially no absorption between 300 and $400 \text{ m}\mu$ before acidification. When this latter solution is acidified, the band at $350 \text{ m}\mu$ again appears. Since it has been shown by other investigators (7, 13) that the bridge compound $f^5 \text{ }^{10}\text{FH}_4$ (λ_{max} at $350 \text{ m}\mu$) is produced *immediately* upon acidification of $f^{10}\text{FH}_4$, but only very slowly from $f^6\text{FH}_4$ (*cf* Equation 5), it would appear that the



product of the hFH_4 dehydrogenase reaction is the N^{10} -formyl isomer of FH_4 .

The amount of $f^{10}\text{FH}_4$ at the completion of a reaction (calculated from the known extinction coefficient of $f^5 \text{ }^{10}\text{FH}_4$) was always considerably less than the amount of TPNH, as shown by two separate experiments (Experiments 1 and 2, Table II). This effect has been shown to be referable to the action of an $f^{10}\text{FH}_4$ deacylase (*cf* Equation 6)



present in *beef liver* preparations. The properties of this enzyme will be discussed in a separate communication. In *pigeon liver* preparations from which the deacylase is absent, there is an exact stoichiometry between the amount of $f^{10}\text{FH}_4$ and TPNH (*cf* Experiments 3 and 4, Table II).

Because pigeon liver extracts are devoid of $f^{10}\text{FH}_4$ deacylase activity, thus permitting the accumulation of $f^{10}\text{FH}_4$, it is possible to demonstrate the effect of FH_4 upon the equilibrium of the coupled reaction involving serine hydroxymethylase and hFH_4 dehydrogenase. For the experiment illustrated in Fig. 3, a Dowex-treated pigeon liver extract was used and

TPNH production was measured Initially L-serine, TPN, and buffer were present, other substances were added at the times indicated by the arrows When both FH_4 and enzyme were added, TPNH production was observed until an equilibrium is reached Addition of fresh enzyme did

TABLE II
Stoichiometry of $f^{10}\text{FH}_4$ and TPNH

Experiment No	Enzyme*	Δ TPNH	$\Delta f^{10}\text{FH}_4$ †
		μmole	μmole
1	Beef liver	0.23	0.09
2	" "	0.42	0.05
3	Pigeon liver	0.51	0.49
4	" "	0.08	0.10

Four separate representative enzyme preparations were tested by using the standard spectrophotometric assay system with the addition of 10 μmoles of 2-mercapto ethanol

* Dowex-treated

† Estimated as f^5 FH_4

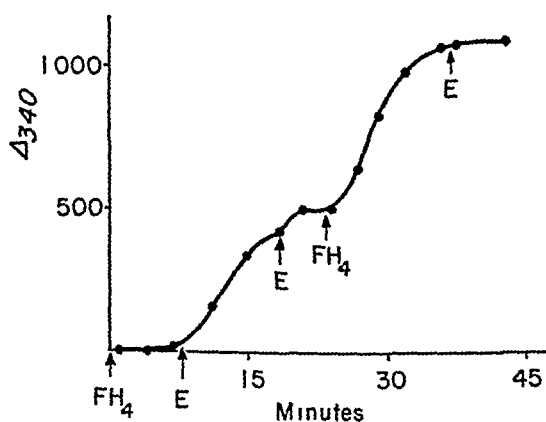


FIG 3 Effect of FH_4 upon equilibrium of coupled reaction The experimental cuvette contained 10 μmoles of L-serine, 1.2 μmoles of TPN, 150 μmoles of phosphate buffer, pH 7.5, 10 μmoles of 2-mercaptoethanol, and water to make 2.6 ml At the indicated points, the following additions were made 0.03 ml of enzyme (E) and 1.2 μmoles of DL- FH_4 L-Serine was omitted from the blank

not appreciably alter the optical density reading However, addition of a second increment of FH_4 (equal to the amount originally added) again initiated the reaction and produced approximately the same amount of TPNH At the conclusion of the experiment, it could be calculated from the net optical density change that a total of 0.51 μmole of TPNH had been formed The amount of $f^{10}\text{FH}_4$, determined as f^5 FH_4 , was found to be 0.49 μmole

In order to determine whether the dehydrogenation was readily reversible and to obtain further evidence for the involvement of the N^{10} -formyl isomer in Equation 2, a spectrophotometric study was made of the conversion of TPNH to TPN in the presence of $f^{10}\text{FH}_4$ (see "Experimental"). In two separate experiments, 0.082 and 0.077 μmoles of TPNH disappeared in 30 minutes in the experimental cuvettes relative to the blank. Under the same conditions, $f^5\text{FH}_4$ was unable to bring about any net disappearance of TPNH. This experiment, in addition to the previous demonstration that $f^{10}\text{FH}_4$ rather than $f^5\text{FH}_4$ is the product of the hFH_4 dehydrogenase reaction, would strongly suggest that the substrate for the enzyme is the N^{10} -hydroxymethyl isomer of FH_4 rather than the N^5 isomer.⁵ However, final elucidation of this must await the unambiguous chemical

TABLE III
Inhibition by p-Chloromercuriphenyl Sulfonate

Inhibitor added	Substrate			
	Serine		HCHO	
	TPNH formed	Inhibition	TPNH formed	Inhibition
μmole	μmole	per cent	μmole	per cent
None	0.14		0.15	
0.2	0.04	72	0.05	67
0.6	0.00	100	0.00	100

Standard spectrophotometric assay systems with amount of inhibitor added as indicated. Time of experiment, 30 minutes.

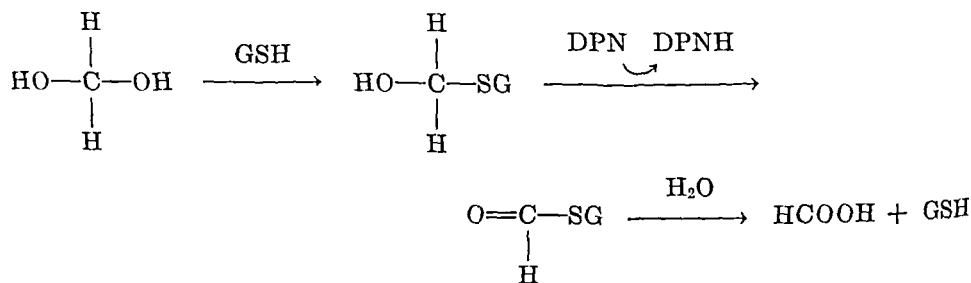
synthesis of the two hydroxymethyl isomers and their assay with a purified hFH_4 dehydrogenase.

Inhibition by p-Chloromercuriphenyl Sulfonate—The hFH_4 dehydrogenase system, as represented by Equations 1 and 2 or Equations 4 and 2, contains a sensitive essential thiol group, as shown by the following data. After dialysis of the enzyme against 1×10^{-3} M PCMB, followed by dialysis against phosphate buffer, TPN reduction in the presence of serine was completely abolished and was not restored by the addition of GSH to the assay system. A control experiment, wherein the enzyme preparation was dialyzed for the same period of time against only phosphate buffer, showed no loss of activity due to prolonged dialysis. The same inhibitory effect could be shown also by the direct addition of PCMPs to

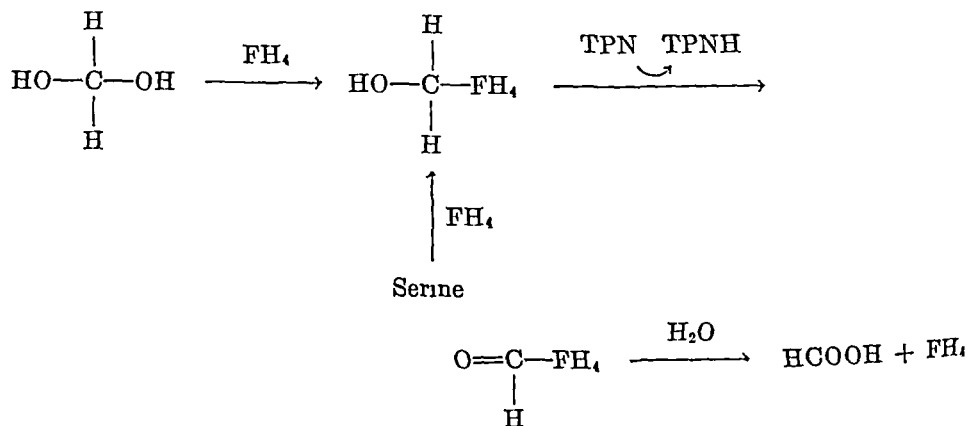
⁵ Note added in proof. Recent experiments have shown that when the dehydrogenase is freed from the contaminating enzyme *cyclohydrolase* (16), which carries out the reaction, $f^5\text{-}^{10}\text{FH}_4 + \text{H}_2\text{O} \rightleftharpoons f^{10}\text{FH}_4 + \text{H}^+$, $f^5\text{-}^{10}\text{FH}_4$, rather than $f^{10}\text{FH}_4$, is the reaction product, conversely, only $f^5\text{-}^{10}\text{FH}_4$ serves as substrate for reversal of the dehydrogenase under these conditions.

the assay system (Table III) Parallel experiments were carried out with serine and HCHO as substrates in the presence and absence of inhibitor Essentially the same degree of inhibition for each substrate was observed with the variation in the amount of inhibitor This experiment suggests that PCMPS inhibition occurs at a site common to the pathway of both substrates, *i e* the dehydrogenase

The above evidence offers an additional point of differentiation between the TPN-linked hFH₄ dehydrogenase and the DPN-linked HCHO dehydrogenase of Stittmatter and Ball (11) Although the latter enzyme in highly purified form requires GSH as a cofactor, we have found that, in crude enzyme preparations, presumably containing endogenous or bound GSH, the enzyme is not stimulated appreciably by added GSH Under conditions of Table III, the addition of PCMPS, at low levels (0.6 μ mole) which caused complete inhibition of the TPN-linked enzyme, did not cause any inhibition of the DPN-linked counterpart The latter enzyme could be inhibited, however, by higher levels of PCMPS Thus, the inhibition experiments provide an additional means of distinguishing between the two formaldehyde dehydrogenases, whose mechanisms may be represented by the following schemes



Formaldehyde dehydrogenase



Serine

Hydroxymethyl tetrahydrofolic dehydrogenase

The authors are indebted to Dr P T Talbert and Dr H R Whiteley for many stimulating discussions and to Mrs Enid Veicamer for her capable assistance on this problem

SUMMARY

1 Hydroxymethyl tetrahydrofolic dehydrogenase has been studied in soluble preparations obtained from beef liver acetone powders Partial purification of the enzyme was achieved by means of precipitation at pH 6.2 and by adsorption and elution from calcium phosphate gel

2 The enzyme catalyzes the reduction of triphosphopyridine nucleotide (but not diphosphopyridine nucleotide) in the presence of the substrate, hydroxymethyl tetrahydrofolate, which may be generated (1) from L-serine and tetrahydrofolic acid with use of the enzyme, serine hydroxymethylase, or (2) from formaldehyde and tetrahydrofolic acid The product is N^{10} -formyl tetrahydrofolic acid, and the reaction is readily reversible

3 The enzyme is inhibited by *p*-chloromercuriphenyl sulfonate, indicating the presence of an essential thiol group

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THE ISOLATION AND DETERMINATION OF URINARY HYDROXYKYNURENINE*

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The enzymatic synthesis of 3-hydroxykynurenine from L-kynurenine by liver and kidney mitochondria was recently demonstrated (1). This *o*-aminophenol is reported to be an intermediate in the conversion of tryptophan to niacin (2, 3), is a precursor of xanthurenic acid (4), and leads to the formation of pigments in insects (5, 6). In certain human pathological conditions it is excreted in elevated amounts in the urine (7, 8) and has been isolated from the urine of patients with hemoblastic disease but was not detected in normal urine (8). 3-Hydroxykynurenine and 3-hydroxyanthranilic acid were recently reported to be present in greater than normal amounts in the urine of patients with bladder cancer (9), and 3-hydroxyanthranilic acid was carcinogenic when implanted into the bladders of mice (10).

In the development of quantitative ion exchange column procedures for the determination of tryptophan metabolites in urine (11), the kynurenine fractions frequently became yellow upon addition of nitrite, and paper chromatograms of these fractions revealed a yellowish green fluorescent spot corresponding to 3-hydroxykynurenine. These observations provided the basis for the development of a method for the determination of 3-hydroxykynurenine in human urine without the use of additional column procedures. The method of determination depends upon the increase in yellow color of 3-hydroxykynurenine in the presence of nitrous acid and is similar in principle to the method recently described by Inagami (12) for the determination of 3-hydroxykynurenine in silkworms except that ion exchange chromatography is used to remove interfering chromogens. The ion exchange procedure was used on a larger scale to isolate 3-hydroxykynurenine in good yield from urine of patients who excreted large quantities of 3-hydroxykynurenine.

EXPERIMENTAL

The materials and apparatus used were those described previously (11). 3-Hydroxy-DL-kynurenine was kindly supplied by Dr. L. M. Henderson.

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Most of the urine samples used for the isolation of 3-hydroxykynurenine were from patients ingesting isoniazid for another study (13)

Determination of 3-Hydroxykynurenine—Fraction E (kynurenine fraction) was obtained as previously described (11) To determine the recovery of 3-hydroxykynurenine from the column, an additional column was used to which was applied a duplicate urine sample with 400 to 800 γ of hydroxykynurenine added 30 ml of Fraction E were pipetted into each of three colorimeter tubes, and 0.2 ml of 0.25 per cent NaNO_2 was added to Tubes 2 and 3 3 minutes after mixing the nitrite, 0.2 ml of 10 per cent ammonium sulfamate was added to all of the tubes The first tube was then made to the same volume as the others by the addition of 0.2 ml of water The optical density at 367 $m\mu$ was then measured in a modified Beckman DU spectrophotometer by using the first tube as a blank A standard curve was run at the same time with hydroxykynurenine in 5 N HCl (10 to 40 γ per tube) From the standard curve, the column recovery and the mg per 24 hour urine sample were calculated

Isolation of 3-Hydroxy-L-kynurenine from Urine—Patients receiving large doses of isoniazid were shown to excrete large amounts of hydroxykynurenine, especially after oral supplementation with 2.0 gm of L-tryptophan (13) From one to four 24 hour urines from such subjects, after ingestion of 2.0 gm of L-tryptophan, were pooled, acidified to 0.1 N with HCl, and passed through a column of Dowex 50 (H^+) 5.5 cm in diameter and 11 cm long The sample was followed by 11 liters of 0.5 N HCl and then by 50 liters of water This large volume of water was shown to remove certain quinoline derivatives (14) The column was then washed with 2.8 liter volumes of 1.0 N, 2.4 N, and 5.0 N HCl The last fraction contained the hydroxykynurenine and gave a strong yellow color reaction with sodium nitrite This fraction was vacuum-distilled to dryness at 25° to 30° under nitrogen, and the sticky brown residue was dissolved in 5 to 10 ml of 0.1 N HCl A few mg of charcoal and of (ethylenedinitrilo)-tetraacetic acid were added, and the suspension was filtered Upon adjusting the filtrate to pH 3.5 with saturated sodium acetate solution, golden yellow needles of 3-hydroxykynurenine appeared The product was allowed to stand in the refrigerator for a few hours before the crystals were centrifuged, washed with a small volume of water, redissolved in a few ml of 0.5 N HCl, and then treated with charcoal and (ethylenedinitrilo)-tetraacetic acid before reprecipitation at pH 3.5 The yield at this point was 60 to 65 per cent of the total hydroxykynurenine present in the urine as indicated by the analytical method described above An additional 5 to 10 per cent could be obtained by concentration of the mother liquors The use of (ethylenedinitrilo)-tetraacetic acid with charcoal was found to remove colored impurities more effectively than either one alone

Paper chromatograms of these products usually indicated the presence

of traces of kynurenine which could not be removed by recrystallization under any of several different conditions. The kynurenine could be removed as its sucrose complex (15) under conditions similar to those described by Weichert (16) for the resolution of deoxy-DL-kynurenine. 100 mg of the impure hydroxykynurenine and 1.2 gm of sucrose were dissolved in 12 ml of water with slight warming. Absolute ethanol (200 ml) was added to this solution and the stoppered flask was allowed to stand at -15° for 1 week. The small crystals which formed on the walls of the flask were removed by filtration, and the filtrate was acidified with HCl and vacuum-distilled under nitrogen to a thick sirup. This was dissolved in a total of 6 ml of water and adjusted to pH 3.5 with sodium acetate solution. The crystals were washed with a few drops of water and ethanol and dried (weight, 73 mg). Paper chromatograms of the product showed a single spot which corresponded to the faster moving component of 3-hydroxy-DL-kynurenine (17). For analysis, this product was recrystallized from 70 per cent ethanol and dried to constant weight *in vacuo* at 60° over P_2O_5 .

RESULTS AND DISCUSSION

The absorption spectra of the isolated 3-hydroxykynurenine in phosphate buffers at pH 2.0, 7.4, and 12.0 were identical with those of synthetic 3-hydroxy-DL-kynurenine at all wave lengths between 210 and 500 $m\mu$.

$C_{10}H_{12}O_4N_2$	Calculated	C 53.57, H 5.40, N 12.50
	Found ¹	" 53.64, " 5.10, " 12.35

The specific rotation of a 0.17 per cent solution in water was $[\alpha]_D^{27} -34.0^{\circ}$. The addition of 2 equivalents of hydrochloric acid changed the specific rotation to $+8.5^{\circ}$.

The method for the determination of hydroxykynurenine gave a linear standard curve for concentrations of from 1 to 33 γ per ml. The recovery of added hydroxykynurenine from the ion exchange columns averaged 96.7 per cent for thirty-seven consecutive determinations (range, 83 to 119 per cent). The other known components of Fraction E, which include kynurenine, kynurenic acid, and xanthurenic acid, did not interfere with the determination. The use of this method in connection with the methods previously described for other tryptophan metabolites (11) makes possible the routine determination of 3-hydroxykynurenine in urine with little additional effort. Under certain conditions this compound has been found to be the chief urinary metabolite of tryptophan (13).

The analytical method indicated that normal human subjects excreted 1 to 6 mg of hydroxykynurenine per 24 hours, in good agreement with the

¹ Analyses by Clark Microanalytical Laboratory, 104½ West Main Street, Urbana, Illinois

values reported by Boyland and Williams (9) In the 24 hour period following a test dose of 20 gm of L-tryptophan, normal subjects excreted 5 to 14 mg It was possible to isolate as much as 70 per cent of the amount of hydroxykynurenine reported by the analytical method when large amounts were present in the urine This suggested that the method is reasonably accurate Attempts to isolate the compound from urine of normal, unsupplemented subjects were not successful, although its presence was readily detected by paper chromatography of concentrates of Fraction E

SUMMARY

A method was described for the determination of 3-hydroxykynurenine in urine based on the increase in color caused by the addition of nitrite to a sample of urine previously purified by ion exchange chromatography The method indicated that normal human subjects excrete 1 to 6 mg of hydroxykynurenine per day and 5 to 14 mg in the 24 hours after the ingestion of 20 gm of L-tryptophan The ion exchange procedure was used on a larger scale to isolate 3-hydroxy-L-kynurenine from the urine of patients who excreted large amounts of this compound

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RELATION OF URIC ACID METABOLISM TO RELEASE OF IRON FROM HEPATIC FERRITIN*

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In a previous study (1) we observed a marked increase in plasma iron of dogs subjected to drastic hypotension in the course of fatal experimental hemorrhagic shock. It was suggested that the origin of the increased plasma iron was storage ferritin of the liver and that the stimulus for its release was liver hypoxia. Anaerobic incubation of ferritin with liver slices resulted in an increase of its ferrous iron content which was now capable of dissociation for combination with iron-binding agents such as α, α' -dipyridyl or the plasma iron-binding protein.

In the present study the mechanism of ferritin iron reduction and the nature of the compounds involved in this reaction have been investigated. It has been found that anaerobic rat liver slices produce large quantities of uric acid, hypoxanthine, and xanthine, which are freely diffusible into the medium. Of these compounds, only uric acid reduces ferritin iron directly. However, in the presence of ferritin, the oxidation of hypoxanthine or of xanthine by xanthine oxidase takes place anaerobically, in this reaction ferritin acts as an electron acceptor and its iron is reduced to the ferrous state. The reduction of ferritin iron is, therefore, brought about both by the dehydrogenase activity of xanthine oxidase and by the accumulated uric acid formed by this enzyme.

EXPERIMENTAL

Crystalline horse spleen ferritin was prepared by a method described in previous studies (2). All tissues used were from Wistar strain female rats, weighing 150 to 200 gm. Tissue slices were prepared as for the usual micro-respiration studies, with the exception that the organs were first perfused *in situ* with ice-cold Ringer-phosphate solution. In the case of the small intestine, the contents were washed with cold Ringer-phosphate solution, the intestine was cut longitudinally and washed again, and then cut into small strips before being weighed. For incubation experiments, 1.0 gm

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of liver slices, 0.5 gm of kidney cortex slices, 0.25 gm of spleen slices, or 0.5 gm of intestinal strips was incubated in 50 ml Erlenmeyer flasks with 5 ml of Ringer's phosphate at 37° with continuous shaking.

Uric acid, xanthine (sodium), and hypoxanthine were obtained from the Schwarz Laboratories, Inc. Uricase (assaying 125 units per ml) and milk xanthine oxidase (assaying 15,000 units per ml) were partially purified preparations from the Worthington Biochemical Corporation, and catalase was a crystalline preparation from the same source and assayed 5000 units per ml. Reduced triphosphopyridine nucleotide (TPNH) cytochrome *c* reductase was a gift from Dr B. Horecker. TPN, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Company. Calf liver xanthine oxidase was a partially purified preparation made according to Kjelley (3). 2-Amino-4-hydroxy-6-pteridine aldehyde was a gift from the American Cyanamid Company.

Measurements of Fe^{++} in Ferritin— Fe^{++} in ferritin was not measured quantitatively, but instead the amount of Fe^{++} bound by α, α' -dipyridyl was determined colorimetrically. The amount of Fe^{++} of ferritin which is bound by dipyridyl represents a comparative measure of its Fe^{++} content and varies with the concentration of the ferritin and the pH of the solution (1), more Fe^{++} is bound at acid pH than at neutral or slightly alkaline pH. In order to conserve ferritin during the following fractionation studies, the dipyridyl reaction was carried out at pH 4.6. For measurement of the reduction of ferritin iron, 1 ml of a stock ferritin solution containing 60 mg of total iron was added to 1 ml of 1 M acetate buffer, pH 4.6, and 1 ml of 0.2 per cent α, α' -dipyridyl dissolved in water. 1 ml of the solution to be tested for reducing activity was then added and the mixture incubated for 1 hour at 37°. 4 ml of a saturated solution of ammonium sulfate, previously adjusted to pH 4.6 with sulfuric acid, were then added in order to precipitate the ferritin, and the mixture was centrifuged. The clear, pink-colored supernatant solution, containing the Fe^{++} -dipyridyl complex, was read in a Klett photocolormeter, with use of a No. 52 filter, against a blank solution which contained all the reagents except ferritin. Fe^{++} content was calculated by comparison with a standard solution of Fe^{++} treated with the same reagents. Reduction of ferritin iron at pH 7.4 was also measured by incubating 1 ml of ferritin with 1 ml of 0.5 M phosphate buffer, pH 7.4, 1 ml of dipyridyl, and 1 ml of the solution to be tested for reducing activity. After 1 hour the reaction mixture was quickly treated with 6 ml of an ice-cold solution containing 1 part acetate buffer, pH 4.6, and 5 parts saturated ammonium sulfate, and the entire mixture was centrifuged. The clear supernatant fluid was read in a colorimeter as above.

Preparation of Ferritin Iron-Reducing Substance from Rat Liver—Rat liver slices were incubated in the proportion of 1 gm of slices to 5 ml of Ringer-phosphate solution for varying periods of time in 100 per cent oxygen or nitrogen. The media were freed from particulate matter by centrifugation, heated in a water bath to 80°, and again clarified by centrifugation. 1 ml aliquots were added to 1 ml of stock ferritin solution, and the mixture was assayed for Fe^{++} at pH 4.6. The results (Table I) demonstrate that no reducing substance is present in the medium in which slices are incubated in oxygen, whereas increasing quantities of ferritin iron-reducing substance appear in the medium in which slices are incubated under anaerobic conditions. Most of the reducing activity appears within 1 hour.

TABLE I

Formation of Ferritin Iron-Reducing Substance by Anaerobic Rat Liver Slices

1 gm liver slices were incubated with 5 ml of Ringer-phosphate solution, pH 7.4, at 37°. Ferritin-ferrous iron measured at pH 4.6 after mixing 1 ml of ferritin with 1 ml of supernatant solution. Values for ferritin iron-reducing activity have been corrected by subtracting Fe^{++} content of ferritin in the absence of reducing substance.

Incubation time	Gas phase	Ferritin iron reduced by supernatant solution
min		$\mu\text{moles Fe}^{++}$ per mmole total ferritin iron
120	Oxygen	0
15	Nitrogen	3.2
30	"	5.0
60	"	7.0
120	"	7.9

Purification of Ferritin Iron-Reducing Substance—35 gm of pooled rat liver slices were incubated in several flasks, with a total of 175 ml of Ringer-phosphate solution, for 90 minutes in nitrogen. The cell-free supernatant fluid was heated to 80° and the coagulated proteins were removed by centrifugation. The clear solution (146 ml) is called Fraction A in Table II, which lists the ferritin iron-reducing activity of this and subsequent fractions in terms of mg of Fe^{++} formed per mg of total N in the fraction. Corrections were made in the calculations of total activity for aliquots removed for analyses. A recovery of more than 100 per cent in subsequent fractions suggests the presence of impurities in Fraction A which resulted in low values for ferritin iron reduction.

Fraction A was treated with a 25 per cent solution of basic lead acetate (about 30 ml), added dropwise, with constant stirring, until precipitation ceased. The mixture was allowed to stand overnight in the refrigerator,

was centrifuged, and the precipitate washed twice with 10 ml each of 1 per cent lead acetate solution. The solution and washings were discarded, since no ferritin iron-reducing activity could be demonstrated after removal of the lead with H_2S and removal of the latter by concentration of the solution. The lead precipitate was extracted five times with 20 ml each of 0.2 N HCl and the combined extracts were centrifuged. The supernatant fluid was then treated with H_2S , the PbS was filtered and the precipitate washed with 0.2 N HCl, and excess H_2S was removed from the filtrate by a stream of nitrogen. The filtrate was evaporated *in vacuo* to dryness at 35–45°, and the residue was taken up in a minimum of water. After neutralization with dilute NaOH and adjustment of the volume to 10 ml, this fraction was labeled Fraction B.

Fraction B was placed on a 2 × 12 cm column of low porosity cation exchange resin (Dowex 50-X12, 50 to 100 mesh) on the H^+ cycle, and the

TABLE II
*Ferritin Iron-Reducing Activity and Uric Acid Content of Fractions
Obtained during Purification Procedure*

Fraction	Ferritin iron reducing activity		Uric acid content	
	mg Fe^{++} formed per mg total N	total mg Fe^{++} formed	mg per mg total N	total mg
A	0.038	1.1	0.098	2.9
B	0.410	1.6	0.668	2.6
C	1.220	1.3	2.550	2.5

column was washed with water (100 ml) until the effluent was neutral to litmus paper. The solution was evaporated *in vacuo* as before, neutralized, and made to 10 ml with water. The material adsorbed on the resin was eluted with 100 ml of N NH_4OH , evaporated to dryness, and dissolved in water. The first effluent, containing material not adsorbed by the resin, contained all of the reducing activity and is referred to as Fraction C.

Identification of Ferritin Iron-Reducing Substance with Uric Acid—The reducing activity of Fraction C, calculated from the total nitrogen content, had been concentrated thirty times. In qualitative tests, Fraction C gave a positive reaction with silver nitrate in alkaline solution and a blue color with the Folin phosphotungstic acid reagent, commonly used for the estimation of uric acid (4). The solution had an absorption spectrum with a maximum identical with that of pure uric acid (Fig. 1). Treatment of aliquots of Fraction C with excess uricase resulted in the complete disappearance of the color reaction with the uric acid reagent, of the ferritin iron-reducing activity, and of the absorption maximum at 292 $\text{m}\mu$.

To make certain that the purification procedure had not eliminated another reducing substance present in the original solution, Fraction A was treated with uricase with the same results as those given above for Fraction C. The values for each fraction in Table II therefore represent "true" uric acid content.

Reduction of Ferritin Iron by Uric Acid—Although, during the isolation procedure, ferritin iron-reducing activity was measured at pH 4.6, reduction by pure uric acid was now measured at both pH 4.6 and 7.4. With 50 γ of uric acid, at pH 7.4, the Fe^{++} content of ferritin rose from 1.3 to

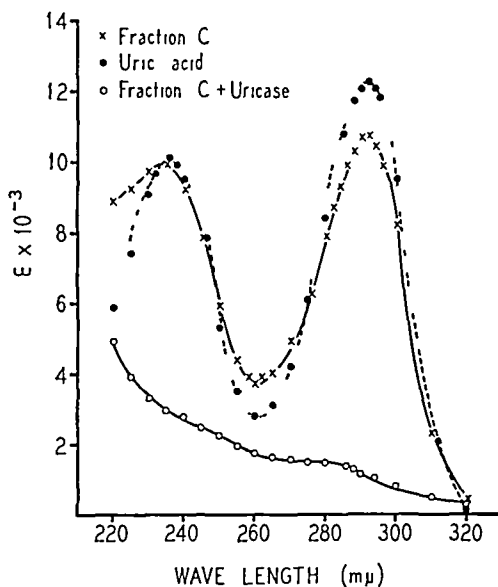


FIG. 1. Absorption spectra of ferritin iron-reducing substance, uric acid, and ferritin iron-reducing substance after treatment with uricase.

2.1 μ moles per mmole of total ferritin iron, at pH 4.6, the increase was from 2.3 to 6.5. This difference reflects the greater availability, at the more acid pH, of ferritin-ferric iron for reduction. At both pH values the extent of reduction was found to vary with the quantity of uric acid added.

Uric Acid Accumulation in Anaerobic Liver—To determine whether an over-all synthesis of uric acid had occurred in the anaerobic liver slice or whether that normally present in the tissue had merely diffused out of the slice under the influence of lowered oxygen tension (lowered permeability), aliquots of liver slices were prepared and incubated, one in nitrogen and another in oxygen, for 1 hour, as described above. In addition, a third (control) aliquot of slices was placed immediately in 10 ml of boiling water for 1 minute. The heated tissue suspension was homogenized and the

cooled homogenate diluted with 0.1 M phosphate buffer, pH 7.4, to 25 ml. The incubated tissues were separated from their media, and each was treated as above. The media were also heated to boiling and aliquots of all specimens analyzed for uric acid as follows. Two equal aliquots of each sample were mixed separately with 2 ml of 0.1 M phosphate buffer, pH 7.4. One aliquot was treated with 0.3 ml of a 1:10 uricase solution and the other with an equal quantity of water. (This quantity of uricase had been shown in preliminary experiments to be far in excess of that required, at pH 7.4, to destroy all uric acid present.) Both solutions were made to 10 ml with water and incubated for 1 hour with shaking at 37°. After

TABLE III

Uric Acid in Rat Tissues after Aerobic or Anaerobic Incubation

The conditions are as described in the text. The values are expressed as micro moles of uric acid per 100 gm of wet tissue or equivalent of solution.

Incubation	Fraction	Liver	Kidney	Spleen	Small intestine
Control (no incubation)	Tissue	12.4	4.1	25.0	32.2
Oxygen	Tissue	8.1	14.6	26.2	51.0
	Medium	0.0	142.0	155.5	377.0
Total		8.1	156.6	181.7	428.0
Nitrogen	Tissue	14.5	7.0	26.8	23.7
	Medium	72.7	30.7	78.5	171.0
Total		87.2	37.7	105.3	194.7

incubation, each solution was treated with 2 ml of 10 per cent sodium tungstate and 1.8 ml of N sulfuric acid, to stop the reaction and to remove protein. 10 ml aliquots of the clear supernatant solutions obtained by centrifugation were mixed with 2.5 ml of urea-cyanide reagent and 1 ml of uric acid reagent, and the colors were compared after 30 minutes, in the Klett photocolormeter, with that produced by a known quantity of uric acid treated in a similar fashion. A No. 66 filter was used. The concentrations of "true" uric acid were calculated by subtracting the values due to reducing non-uric material remaining after uricase treatment from the totals. In this way the values were obtained for the uric acid content of control tissue, tissue and medium after oxygen incubation, and tissue and medium after nitrogen incubation. The results (Table III) demonstrate for the liver a marked accumulation of uric acid in the anaerobic slices. The extra uric acid was diffusible into the medium.

Effect of Oxygen Tension on Uric Acid Accumulation in Liver Slices—The relationship between oxygen tension and uric acid production by the liver *in vitro* was studied in a manner similar to that described above, except that the oxygen content of the gas phase was varied. After the incubation mixtures were heated to 80°, the clear supernatant media were analyzed. Accumulation of uric acid in liver slices occurred at oxygen tension of air (20 per cent) and increased markedly at lower tensions. Amounts of uric acid formed per 5 ml. of medium were 7 γ at 20 per cent, 35 γ at 10 per cent, and 100 γ at zero per cent oxygen (tank nitrogen).

TABLE IV
*Formation of Uric Acid and Hypoxanthine Plus Xanthine during
Aerobic or Anaerobic Incubation*

The conditions are as described in the text. The results are expressed as micro-moles per organ in the rat, wet weights of organs were 8.5 gm. of liver, 1.0 gm. of spleen, 2.2 gm. of kidneys, and 6.6 gm. of small intestine.

Tissue	No incubation	Oxygen	Nitrogen
Liver			
Uric acid	1.05	0.69	7.40
Hypoxanthine + xanthine	1.62	0.76	25.60
Spleen			
Uric acid	0.20	1.38	0.71
Hypoxanthine + xanthine	0.22	0.29	2.18
Kidneys			
Uric acid	0.09	3.45	0.83
Hypoxanthine + xanthine	0.53	3.88	9.74
Small intestine			
Uric acid	2.13	28.25	12.85
Hypoxanthine + xanthine	3.26	3.46	23.90

Uric Acid Production in Other Rat Tissues—The accumulation of uric acid in kidney cortex, spleen, and small intestine was measured in both oxygen and nitrogen in a manner similar to that described above for liver. The data (Table III) show that uric acid is formed in these three tissues in the presence and the absence of oxygen. However, only in the liver does more uric acid accumulate anaerobically than aerobically. These results also reveal that aerobic conditions produce high uric acid levels per gm. of kidney, spleen, and especially intestine. To show the relationship of these values in the whole animal, the results in Table IV have been calculated in terms of the contributions of whole wet weight organs.

Anaerobic Accumulation of Hypoxanthine and Xanthine in Rat Tissues—Since, so far as is known, the formation of uric acid in rats is dependent upon oxidation, by xanthine oxidase, of hypoxanthine and xanthine, these

compounds were estimated in tissues treated as above. For these experiments, instead of analysis of the incubated tissue and medium separately, the entire incubation mixture was heated and homogenized, and aliquots were removed for analyses. The "true" uric acid content of one aliquot of the homogenate was determined by the uricase procedure. Two additional aliquots were treated with excess xanthine oxidase to convert all of the xanthine and hypoxanthine to uric acid. After this, one sample was treated with excess uricase to destroy the uric acid present originally, as well as that formed from hypoxanthine and xanthine by the action of xanthine oxidase. The quantity of uric acid formed from the xanthine and hypoxanthine could now be calculated by subtraction of the original uric acid content and correction for any reducing material remaining after uricase treatment. Table IV shows that in all tissues anaerobiosis caused a marked increase in concentration of xanthine and hypoxanthine. To determine the relative amounts of hypoxanthine and xanthine present in liver, the spectrophotometric method of Kalckar (5) was employed in one experiment. They were found to be present in approximately equal quantities.

Of the four tissues studied in the rat, uricase was found to be present only in the liver. Uricase activity was determined by the method of Leone (6) and was equal to 162 μ l of O_2 per 100 mg of wet weight of tissue per hour at 37°.

Effect of Oxygen Tension on Uricase and Xanthine Oxidase Activities—In order to explore the factors influencing uric acid accumulation in anaerobic liver, the sensitivities of the enzymes involved in uric acid metabolism to lowered oxygen tension were measured with use of partially purified enzymes. In the uricase experiments, each flask contained 2 ml of 0.1 M glycine buffer, pH 9.4, 0.5 ml of a 1:10 uricase solution, and 2.45 ml of water. A neutralized solution of 60 γ of uric acid in a volume of 0.5 ml was suspended, in a small plastic cup, from a rubber stopper which was also fitted with inlet and outlet tubes used for the appropriate gas mixture. After preliminary incubation for temperature and gas equilibration, the inlet and outlet tubes were clamped and the cup containing uric acid was tipped into the reaction mixture. After 15 minutes, the reaction was stopped by addition of tungstate and sulfuric acid and the solution analyzed for uric acid, as described previously. In xanthine oxidase experiments, xanthine or hypoxanthine was used as substrate. Reaction mixtures contained 2 ml of 0.1 per cent albumin in 0.1 M phosphate buffer, pH 7.4, 0.03 ml of a 1:25 xanthine oxidase solution, and 2.72 ml of water. The substrate, containing 250 γ of hypoxanthine, or an equivalent quantity of xanthine, in 0.25 ml, was suspended in the plastic cup. The xanthine reaction was allowed to continue for 15 minutes and the hypoxanthine

reaction for 75 minutes before analyses for uric acid were made. Fig 2 shows the relative activities of the enzymes at various oxygen tensions, with an activity of 100 representing that of the respective enzyme in 100 per cent oxygen. Uricase is affected to a greater extent than xanthine oxidase as the oxygen tension is lowered. When xanthine is used as a substrate, xanthine oxidase activity is somewhat inhibited by oxygen tensions above 60 per cent. A similar inhibition of xanthine oxidase activity by high oxygen pressure has been reported by Stadie and Haugaard (7).

Ferritin As Electron Acceptor for Xanthine Oxidase—Xanthine oxidase can act as a dehydrogenase in the presence of electron acceptors other than

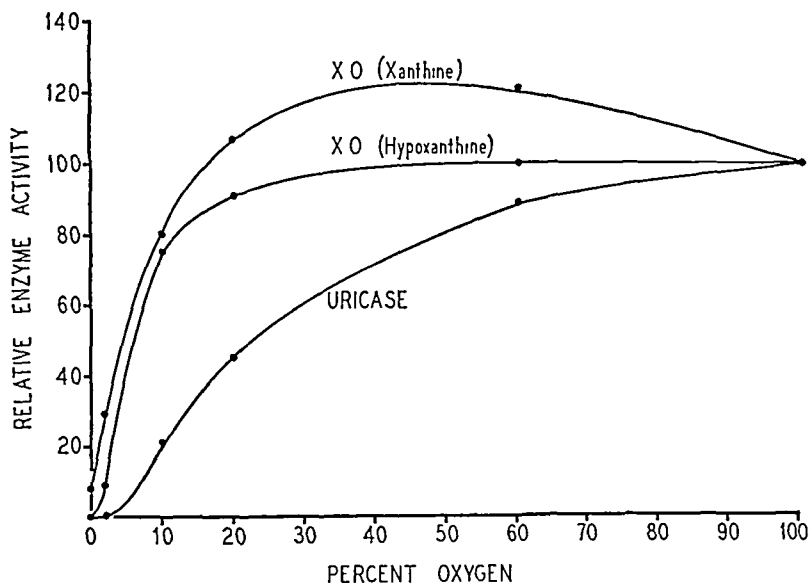


Fig 2 Effect of oxygen tension on activities of xanthine oxidase and uricase

molecular oxygen, such as methylene blue (8) or ferricytochrome *c* (9). In liver slice experiments, uric acid diffuses out of the anaerobic cells and reduces ferritin in the medium, but, in the intact liver, uric acid would be synthesized inside the cell in the presence of ferritin. We therefore explored the possibility that the process of uric acid formation, involving the action of xanthine oxidase, might bring about ferritin iron reduction, in which case the ferric iron in ferritin would act as the electron acceptor for the enzyme. For this purpose, reaction mixtures were prepared consisting of 1 ml of ferritin previously adjusted to pH 7.4 with dilute alkali and containing 6.13 mg of total iron, 1 ml of 0.2 per cent α, α' -dipyridyl to act as a trapping agent for any Fe^{++} formed, 1 ml of 0.1 M phosphate buffer, pH 7.4, 0.03 ml of 1.25 M xanthine oxidase, and water to make a final volume of 4 ml. A suspended plastic cup contained 250 γ of xan-

thine in 0.25 ml or an equivalent quantity of hypoxanthine. After temperature and gas equilibration, the reaction was started by tipping the cup and its contents into the reaction mixture. At appropriate intervals, 6 ml of a chilled mixture, containing 1 part of 1 M acetate buffer, pH 4.6, and 5 parts of saturated ammonium sulfate previously adjusted to pH 4.6, were added to each flask. The mixture was centrifuged and the supernatant fluid compared in the colorimeter with a Fe^{++} standard treated in the same manner.

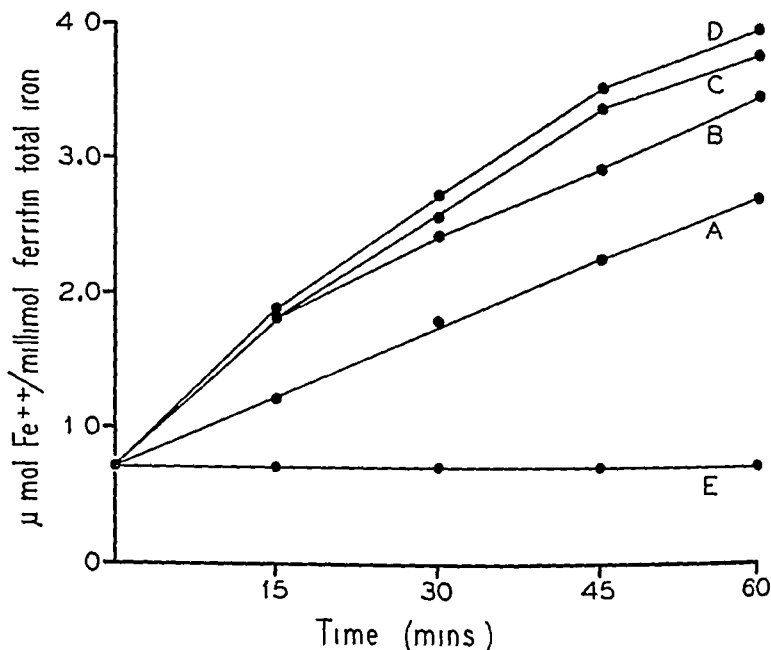


FIG. 3. Action of ferritin iron as electron acceptor in the xanthine oxidase system. Curve A, ferritin reduction by enzyme in absence of oxygen, Curve B, ferritin reduction by enzyme in presence of 100 per cent oxygen, Curve C, same as Curve B plus 0.05 mg of crystalline catalase, Curve D, same as Curve B plus 0.5 mg of crystalline catalase, Curve E, same as Curve A or Curve B plus 2.25 γ per ml of 2-amino-4-hydroxy-6-pteridine aldehyde.

The results obtained with hypoxanthine as a substrate are shown in Fig. 3. Appreciable reduction of ferritin iron occurs in the complete absence of oxygen as indicated by Curve A. The direct reaction of ferritin with xanthine oxidase is apparent from the fact that, in the absence of ferritin, no uric acid is formed anaerobically. More ferritin iron was reduced when the reaction was carried out in the presence of 100 per cent oxygen (Curve B). In addition, experiments carried out with hypoxanthine as well as xanthine as substrates, in the presence of oxygen tensions between zero and 100 per cent, yielded values for ferritin iron reduction intermediate between those obtained in 100 per cent nitrogen and in 100 per cent oxygen. Similar results were also obtained with xanthine ox-

idase prepared from calf's liver by the method of Kielley (3). Curves C and D in Fig. 3 demonstrate that the aerobic reduction of ferritin iron is increased by the addition of crystalline catalase over a 10-fold range of concentration (0.05 mg and 0.5 mg, respectively, of crystalline enzyme), suggesting that xanthine oxidase activity is inhibited by peroxide formed during the reaction (10). That xanthine oxidase was responsible for ferritin iron reduction was made more certain by our finding that this reduction was completely inhibited (Curve E) in the presence of 2.25 γ per ml of 2-amino-4-hydroxy-6-pteridine aldehyde (11). This inhibition occurred in both nitrogen and oxygen.

Reduction of Ferritin Iron by TPNH Cytochrome c Reductase—Weber *et al.* (12) have shown that several flavoprotein enzymes are capable of reducing inorganic ferric iron in the presence of citrate, among them xanthine oxidase and TPNH cytochrome *c* reductase. Since cytochrome *c* reductase is also found in liver, experiments were performed *in vitro* to investigate its ability to reduce ferritin iron. The reaction mixture contained 1 ml of ferritin, 1 ml of 0.2 per cent dipyridyl, 1 ml of 0.1 M phosphate buffer, pH 7.55, 0.1 ml of glucose 6-phosphate (1.65 mg), 0.02 ml of glucose-6-phosphate dehydrogenase (0.2 mg), 0.05 ml of triphosphopyridine nucleotide (25 γ), and water to make a final volume of 4 ml. The reaction was started by the addition of 0.05 ml of a partially purified cytochrome *c* reductase preparation. The purpose of the glucose 6-phosphate and its dehydrogenase was to insure a constant source of TPNH, as in an assay method described by Haas (13). The original ferritin, assayed at pH 7.4, contained 0.45 μ mole of Fe^{++} per mmole of total iron. In the absence of the cytochrome *c* reductase, the TPNH generated by the dehydrogenase system itself caused an increase in Fe^{++} to 0.56 μ mole. Upon addition of the reductase, the Fe^{++} content rose to 1.22, in 100 per cent oxygen as well as in 100 per cent nitrogen. However, TPNH in rat liver slices was found to be lower after anaerobic incubation (16 γ per gm of wet weight) than after aerobic incubation (57 γ per gm), decreasing the likelihood that cytochrome *c* reductase is important for ferritin iron reduction.

Plasma Uric Acid of Rats in Hemorrhagic Shock—The results of our experiments performed *in vitro* suggest that the plasma uric acid level should increase as a result of liver hypoxia in animals subjected to hemorrhagic shock. Zweifach *et al.* (14) and Van Slyke (15) reported increased plasma uric acid concentrations in dogs in hemorrhagic shock. In a series of experiments, to be reported in detail at a later date, we have confirmed these findings for the rat in hemorrhagic as well as in traumatic (drum) (16) shock, and for the dog in hemorrhagic shock¹. Since the drastic hypo-

¹ We are indebted to Dr. Baez, Dr. Srikantha, and Anne Carleton for providing us with blood samples from their rats and dogs in hemorrhagic shock for these uric acid analyses.

tension induced in the hemorrhagic shock experiment could be presumed to result not only in liver hypoxia but in renal failure as well, it was necessary to determine whether the observed rise in plasma uric acid could be attributed to renal failure. For this purpose plasma was collected 3 hours after bilateral nephrectomy of twelve control rats. Plasma uric acid, expressed as mg per 100 ml, averaged 0.6 (range 0.3 to 1.1) in eleven normal control rats, in the twelve arenal controls the average was 2.4 (range 1.1 to 2.8), and in eleven normal rats subjected to hemorrhagic shock the values averaged 8.7 (range 4.9 to 14.5).

DISCUSSION

In our earlier experiments, designed to study the mechanism by which ferritin iron is reduced, liver slices were incubated anaerobically in a medium containing ferritin (1). Under these conditions, reduction of ferritin iron could only be a result of diffusion into the medium of a compound of low molecular weight, since it would not be reasonable to expect that ferritin would enter the cell, be reduced, and then diffuse out into the medium. The reducing compound produced by anaerobic liver slices has now been identified as uric acid, and its concentration has been found to increase as oxygen tension is decreased to 20 per cent and below. *In vivo*, reduction of ferritin iron would take place inside the hepatic cell in the presence of hypoxanthine, xanthine, and the enzyme, xanthine oxidase, which converts these substrates to uric acid.

Xanthine oxidase is an iron molybdoflavoprotein (17) classified as an aerobic dehydrogenase, since it can utilize molecular oxygen as an electron acceptor. In addition, methylene blue and ferricytochrome *c* react with this enzyme and are reduced. Ferritin iron can also act as an electron acceptor for reduced xanthine oxidase, and does so even in the complete absence of oxygen. The anaerobic reduction of ferritin iron is therefore primarily due to the enzyme and only secondarily to the uric acid which is formed. The presence of oxygen in the xanthine oxidase system increases ferritin iron reduction, but it is not possible to estimate how much of the reduction is due to reaction with the reduced enzyme and how much to the increased quantity of uric acid formed aerobically. Although it is assumed that ferritin iron is reduced by reaction with reduced flavin adenine dinucleotide of the enzyme, the possibility of reaction with the Fe or Mo of the enzyme still exists. In fact, Mackler *et al* (18) have demonstrated that, although removal of Mo from the enzyme does not inhibit the reaction of the enzyme with molecular oxygen, it does inhibit its reaction with 1 electron acceptors.

Our results for the reduction of ferritin iron parallel those of Weber *et al* (12) for the reduction of ferric citrate by xanthine oxidase, since in both

cases reduction is stimulated by the addition of oxygen, but Weber's reaction is inhibited in the presence of catalase whereas that of ferritin iron is stimulated, suggesting that in the latter reaction peroxide acts as a xanthine oxidase inhibitor. A further difference in the two reactions is that under anaerobic conditions reduction of ferritin iron is considerable, whereas Weber *et al* find that little reduction of ferric citrate occurs anaerobically. Both ferric citrate and ferritin iron are reduced by TPNH cytochrome *c* reductase, however, it appears unlikely that this flavoprotein enzyme plays a significant role in the reduction of ferritin iron *in vivo* since TPNH levels in rat liver slices were found to be lower after anaerobic than aerobic incubation. The possibility of reduction by other flavoprotein enzymes is not ruled out.

Tanaka (19) also reported that, in the presence of hypoxanthine, xanthine oxidase reduced ferritin iron anaerobically. Ferrous iron was not measured directly in this study. Instead, measurements were made of changes in magnetic susceptibility of ferritin. However, the data given by this author are contradictory in that the values reported for susceptibility increased after treatment of ferritin with ascorbic acid and cysteine and decreased after its incubation with xanthine oxidase. We have repeated these experiments² and have found that treatment of ferritin with the xanthine oxidase system produces a rise in specific susceptibility of ferritin iron, a result to be expected from the studies of Michaelis *et al* (20), who first pointed out the unusual state of iron in the ferritin molecule and the effect of reducing agents such as $\text{Na}_2\text{S}_2\text{O}_4$.

Several factors apparently influence the accumulation of hypoxanthine plus xanthine and of uric acid in the various tissues subjected to aerobic or anaerobic incubation *in vitro*. In all four tissues examined, liver, spleen, kidney, and small intestine, the concentrations of hypoxanthine plus xanthine increased markedly in response to lowered oxygen tension. These increases can be attributed to the progressive loss of high energy compounds needed for synthetic reactions involving the purines (21) and to an increased effectiveness of the catabolic enzymes acting on nucleotides and nucleosides (22). Thus, the anaerobic sources of hypoxanthine and xanthine may be purine derivatives, not as yet incorporated into nucleic acids, as well as degraded nucleic acids. Jorgensen and Poulsen (23) have reported a sharp rise in hypoxanthine and xanthine in stored red cells, in our experiments this source was eliminated by thorough perfusion of the organs before use.

In the liver, uric acid accumulates during anaerobic incubation and decreases slightly upon aerobic incubation as compared with unincubated

² We are indebted to Dr D Rittenberg, Department of Biochemistry, Columbia University, for the use of his Gouy balance for the susceptibility measurements.

controls Similar results have been reported by Bernheim and Bernheim (24) The anaerobic increase can be accounted for by the action of xanthine oxidase on the higher concentration of uric acid precursors together with anaerobic inhibition of the enzyme uricase In the aerobic rat liver slice the decrease in uric acid is doubtless due to uricase action In the non-hepatic tissues, on the other hand, we found less anaerobic than aerobic accumulation of uric acid A possible explanation for this difference may be found in the experiments of Westerfeld and Richert (25), who have shown that the dehydrogenase activity (as measured by the increase in O_2 uptake in the presence of methylene blue) of the xanthine oxidases in these non-hepatic tissues is lower than that in rat liver

The results of our experiments *in vitro*, together with those which demonstrate increased concentrations of both uric acid and iron (1) in the plasma of animals in hemorrhagic shock, support the hypothesis that the xanthine oxidase system plays an important role in the process of iron reduction and release from hepatic ferritin When the liver is supplied with adequately oxygenated blood, iron incorporation is accomplished by the withdrawal of iron from the plasma, as shown by experiments with radio active iron (26) Under the same conditions (as demonstrated by our experiments *in vitro* at 20 per cent oxygen tension) small amounts of ferritin iron can be reduced and released into the plasma, at a rate consistent with the requirements of the hematopoietic system

Our results emphasize lowered oxygen tension as a stimulus for increased formation of hypoxanthine, xanthine, and uric acid in the liver In addition, tissues such as the spleen and small intestine also yield relatively large quantities of uric acid precursors which can be metabolized by the liver These increases are reflected by an increased reduction and release of ferritin iron to the plasma via combination with the plasma iron-binding globulin, and explain the mechanism of release of extra iron to the plasma of animals in hemorrhagic shock The response of the animal to lowered oxygen tension, outlined above, helps to explain the origin and mechanism for the release of extra iron needed by the bone marrow during the development of polycythemia at high altitudes Since the life span of the red cell in a polycythemic animal is normal, the rate of iron release from ferritin stores must be increased in order to maintain a flow of iron, via the plasma, to the activated bone marrow Our findings are substantiated by the frequency with which elevated plasma uric acid values occur in patients with polycythemia (27) and by the occasional occurrence of gout, secondary to prolonged polycythemia (28) Here, the increased uric acid, which must arise by virtue of the action of xanthine oxidase, may be a result of the degradation of large quantities of red cell nucleic acids, and of nuclei which are removed from the preerythrocyte cells of the bone

marrow Now under investigation is the occurrence of the xanthine oxidase-ferritin system in bone marrow, spleen, and human placenta, organs which are known to contain ferritin, as well as the implications of these findings for various derangements of iron metabolism in animals

SUMMARY

The reduction (and release) of ferritin iron during anaerobic incubation of ferritin with rat liver slices is due to the accumulation of uric acid in the tissue and its diffusion into the medium Accumulation in anaerobic liver of uric acid precursors, hypoxanthine and xanthine, together with the marked sensitivity to low oxygen tensions of uricase as compared with xanthine oxidase, accounts for the elevated levels of uric acid

Xanthine oxidase, prepared from milk or calf liver, is also capable of reducing ferritin iron under anaerobic conditions in the presence of hypoxanthine or xanthine Reduction is increased in the presence of oxygen and by addition of catalase Ferritin iron reduction is due to the activity of xanthine dehydrogenase, the iron of ferritin acting as an electron acceptor

Although other flavoprotein enzymes can reduce ferritin iron, *e g* reduced triphosphopyridine nucleotide cytochrome *c* reductase, the role of xanthine dehydrogenase in the release of ferritin iron *in vivo* is substantiated by findings obtained with intact animals Rats subjected to hemorrhagic hypotension show abnormally high concentrations of uric acid in the plasma These results, together with the increases in plasma iron, reported previously for dogs in hemorrhagic shock, serve to relate the xanthine dehydrogenase system with the iron release mechanism

The relationship of liver xanthine dehydrogenase, acting as a reducing agent for ferritin iron, to the release of iron into the plasma for extra hemoglobin synthesis by the bone marrow, under conditions of low oxygen tension, is discussed

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STUDIES OF OXIDATIVE PHOSPHORYLATION BY HEPATIC MITOCHONDRIA FROM THE DIABETIC CAT*

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Reports from the laboratories of several investigators suggest the existence of a possible relationship between the action of insulin and the generation of high energy phosphate bonds. In general these studies were designed to determine whether the formation by various tissues of the primary high energy phosphate compound, *i.e.* ATP,¹ or the production of secondary phosphate compounds formed by transphosphorylation reactions involving ATP, *e.g.* the hexose phosphate esters, is diminished in the diabetic state or increased by the addition of insulin either *in vitro* or *in vivo*. For example, Kaplan and Greenberg (1, 2) reported an increased turnover of inorganic isotopic phosphate in various organic phosphate compounds of liver and muscle after insulin treatment. In similar studies Sacks (3) reported that the rate of turnover of labile P of ATP and glucose 1-phosphate in the muscle of the diabetic animal was diminished when compared to that of the normal animal. In another type of experiment the rates of reactions presumably dependent upon the rate of formation of ATP were studied. Such a reaction, for example, is the acetylation of ingested *p*-aminobenzoic acid. Charalampous and Hegsted (4) reported that this reaction was depressed in the intact alloxan-diabetic rat. Accordingly, they concluded that a primary deficiency of ATP formation is the cause of this metabolic defect. Stadie (5) has reviewed in detail other aspects of this subject.

The mitochondria are believed to be the main if not the sole site of the generation of ATP by oxidative phosphorylation. If impairment of oxidative phosphorylation in the intact animal exemplified by the cases cited above is a characteristic of the diabetic state, it is possible that the mito-

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¹ The following abbreviations are used: ATP = adenosine triphosphate, ADP = adenosine diphosphate, P_i = inorganic phosphate, G-6-P = glucose 6-phosphate, and TPN = triphosphopyridine dinucleotide.

chondria isolated from tissues of the diabetic animal might show similar impairment of oxidative phosphorylation when compared with mitochondria similarly prepared from the tissues of normal animals. The present paper reports such comparative studies on mitochondrial preparations from the livers of normal and depancreatized cats.

In earlier unpublished experiments, we attempted to demonstrate differences in the oxidative phosphorylation capacity of mitochondria prepared from the livers of normal and alloxan-diabetic rats. Our data showed no difference. Parks, Adler, and Copenhaver (6) subsequently published data showing no difference in the rate of formation of ATP by similar mitochondrial preparations from normal and alloxan-diabetic rats, a result with which our unpublished work completely conforms.

The possibility, however, that depancreatized cats are more insulin-free than alloxan-diabetic rats led us to repeat the work, using the cat as the experimental animal. Mitochondria isolated from the livers of these cats were equilibrated with suitable media to which was added an oxidizable substrate (pyruvate) to furnish the energy for the conversion of inorganic phosphate into high energy phosphate, according to the reaction, $\text{ADP} + \text{P}_i \rightarrow \text{ATP}$. The newly formed energy-rich phosphate was trapped as glucose 6-phosphate by the addition of hexokinase to the equilibration medium, *viz* $\text{ATP} + \text{glucose} \rightarrow \text{G-6-P} + \text{ADP}$. The rate of ATP formation was calculated either from the rate of disappearance of inorganic phosphate or the rate of formation of glucose 6-phosphate.

Methods

Adult normal and depancreatized cats weighing between 2 and 3.5 kilos were used. In the diabetic cats, complete removal of the pancreas was verified by postmortem examination, postoperative glycosuria equal to or greater than 2.5 gm. per kilo of body weight per 24 hours was usually observed. Diet in all instances was 100 gm. of ground horse meat daily. The normal cats were used after a 24 hour fast, and the diabetic cats 3 days after pancreatectomy. Since the depancreatized animals do not eat, two of the normal cats (Table II) were treated to sham operation and fasted for 3 days before study. The results in these cats appear to be no different from those obtained on the non-operated cats fasted for 24 hours.

Mitochondrial Study Techniques—Mitochondria were prepared by the method of Schneider (7), except that the mitochondrial fraction was washed twice instead of three times. Care was taken to exclude the "fluffy layer" as completely as possible. Each mitochondrial preparation was examined for homogeneity with a phase microscope.

Oxygen uptake was determined by standard manometric methods. The main manometric vessel contained 1.4 ml. of medium (KCl, 0.1 M, potas-

sium phosphate, pH 7.4, 0.007 M, sodium salt of ATP, 0.002 M, cytochrome c, 10^{-5} M, potassium pyruvate, 0.035 M), and 0.5 ml of mitochondrial suspension containing approximately 8.0 mg of protein in 0.25 M sucrose. The side arm contained 0.3 ml of 0.1 M KCl containing 4 mg of hexokinase and 26 μ moles of glucose (phosphate acceptor system).

After equilibration at 30° for 10 minutes, the reaction was initiated by tipping the contents of the side sac into the main vessel, after 20 minutes the reaction was terminated by immersion of the manometric vessel for about 30 seconds in boiling water. Pyruvate was used as the oxidizable metabolite because preliminary observations showed the differences to be most marked with this substrate between tissue from the normal and the diabetic animal. During the reaction inorganic phosphate incorporated into ATP accumulates as glucose 6-phosphate. The rate of oxidative phosphorylation was therefore calculated from the rate of inorganic phosphate disappearance (determined colorimetrically (8)), or the rate of G-6-P formation (determined spectrophotometrically with G-6-P dehydrogenase and TPN (9)). The amount of hexokinase added was sufficient to phosphorylate glucose at a rate 3 to 4 times that of the maximally observed phosphorylation rate of the mitochondria. Errors from ATPase action were thus minimized. The results were expressed in terms of mitochondrial protein, calculated on the assumption that the protein contained 16 per cent of nitrogen which was determined by the microdiffusion technique of Seligson and Seligson (10).

Phosphatase Control—Phosphatase activity of the mitochondrial preparations is a possible source of error in the measurement of the phosphorylation rate when a glucose-hexokinase system is used as a phosphate acceptor, since the phosphatase may decrease G-6-P accumulation to an unknown extent. Accordingly, mitochondrial preparations were prepared from both normal and diabetic cats in exactly the same way as those for the assay of oxidative phosphorylation. The phosphatase activity of these preparations was then measured as follows. In the main vessel were 1.0 ml of 0.1 M KCl containing 5.0 μ moles of potassium phosphate at pH 7.4 and 0.5 ml of mitochondrial suspension containing about 8.0 mg of mitochondrial protein in 0.25 M sucrose, and in the side sac were 20 μ moles of glucose 6-phosphate in 0.7 ml of 0.1 M KCl.

After 10 minutes equilibration, the reaction was initiated by tipping in the contents of the side sac. After 30 minutes further equilibration, the reaction was terminated by immersion in boiling water. The contents of the blank vessels were identical except for the omission of G-6-P. Inorganic phosphate formed from glucose 6-phosphate by phosphatase action was calculated from the difference between the initial and the final total inorganic phosphate, making appropriate corrections for the blank. Data

on the phosphatase activity of mitochondria prepared as described were obtained from four normal and two depancreatized cats. The magnitude of the phosphatase activity of these mitochondrial preparations (mean, 10 μ moles of G-6-P hydrolyzed per gm of protein per minute) was small in comparison with the total phosphorylative capacity, furthermore, the statistically insignificant difference between the normal and diabetic values makes this possible source of error negligible in the interpretation of our data on oxidative phosphorylation by mitochondria.

DISCUSSION

The data obtained from this study of hepatic mitochondria prepared from the livers of depancreatized cats are shown in Table I in three cate-

TABLE I
Oxidative Phosphorylation, Mitochondria from Depancreatized Cat Livers

Experiment No	ATP formation, μ moles P per gm mitochondrial protein per min	Oxygen uptake, micro atoms per gm mitochon- drial protein per min	P/O ratio
80	6	10	0.6
83	17	18	1.0
85	9	8	1.1
87	38	24	1.6
89	17	20	0.9
93	27	19	1.4
Mean	19 ± 4.8	17 ± 2.5	1.1 ± 0.14

gories (a) oxidative phosphorylation rate as calculated from the rate of ATP formation, (b) oxygen uptake, and (c) the P/O ratio. In Table II are given similar data obtained by using hepatic mitochondria from normal cats. For comparison, Table II summarizes the means of both Tables I and II together with their standard error of the mean. From these values the mean differences in the three categories of data are given, together with their standard deviations. The last line (Table II) gives the *P* values for these differences. From these comparative studies on the oxidative phosphorylative capacities of the diabetic and the normal cats, we conclude that the mitochondria from the livers of diabetic cats have significantly lower capacities for oxidative phosphorylation than those from livers of normal cats.

In Tables III and IV are data in which the comparative study of the hepatic phosphorylative ability of livers of normal and diabetic cats was made in a different way. First, a liver biopsy was performed on the cat 24 to 48 hours after removal of the pancreas and a small sample (2 to 4

TABLE II
Oxidative Phosphorylation, Mitochondria from Normal Cat Livers

Experiment No	ATP formation, μ moles P per gm mitochondrial protein per min	Oxygen uptake, micro atoms per gm mitochondrial protein per min	P O ratio
S1	37	17	2.2
S4	164	59	2.8
S6	119	36	3.3
S8	33	19	1.7
98A	31	22	1.4
98B*	90	31	2.9
99	70	36	2.0
112	107	41	2.6
158*	141	52	2.7
Mean, normal	88 ± 16.3	35 ± 4.8	2.4 ± 0.21
" diabetic	19 ± 4.8	17 ± 2.5	1.1 ± 0.14
" difference, normal and diabetic	69 ± 17.0	18 ± 5.4	1.3 ± 0.24
t	4.0	3.4	5.4
P	<0.01	<0.01	<<0.01

* Fasted animals treated to sham operation

TABLE III
*Oxidative Phosphorylation, Mitochondria from Biopsies of Livers of Diabetic Cats**

Experiment No	Cat No	ATP formation μ moles P per gm mitochondrial protein per min	Oxygen uptake, micro atoms per gm mitochondrial protein per min	P O ratio
93	II	27	20	1.4
94	III	51	29	1.8
101	V-1	31	27	1.2
105	V-3	76	36	2.1
107	VI	11	18	0.6
108	VII	11	18	0.6
109	VIII	13	17	0.8
113	IX	61	49	1.2
Mean		35 ± 8.8	27 ± 3.9	1.2 ± 0.19

* Cat V V-1, liver biopsy in diabetic state, V-2, liver biopsy after first insulin treatment, V-3, liver biopsy after relapse to diabetic state, and V-4, liver biopsy after second insulin treatment

gm) of the liver was obtained. Mitochondria were prepared from this sample and assayed in the three categories enumerated in Tables I and II. After vigorous treatment of the cat with insulin, which resulted in a restora-

tion of the cat to an essentially normal status as judged by the level of glycemia and blood sugar, a second liver biopsy was obtained from which mitochondria were prepared as before. Originally it was planned to assay the mitochondria from each cat first in the diabetic state and then after restoration by insulin. In other words each cat would furnish its own control. But the high mortality and the difficulties in achieving appropriate restoration to normal by insulin treatment in all cases prevented us from accomplishing this. We were successful, however, with three cats

TABLE IV
*Oxidative Phosphorylation: Mitochondria from Biopsies of Livers of Insulin-Treated Diabetic Cats**

Experiment No.	Cat No.	ATP formation, μmoles P per cm mitochondrial protein per min	Oxygen uptake microatoms per cm mito- chondrial pro- tein per min	P/O ratio
90	I	202	76	2.7
102	IV	146	59	2.5
104	V-2	50	26	1.9
106	V-4	142	56	2.5
110	VIII	120	46	2.6
114	IX	68	37	1.8
115	X	95	41	2.3
Mean treated		118 ± 19.5	49 ± 6.1	2.3 ± 0.14
“ untreated		35 ± 8.8	27 ± 3.9	1.2 ± 0.19
“ difference treated and untreated		83 ± 21.4	22 ± 7.2	1.1 ± 0.24
t		3.9	3.0	4.6
P		<0.01	<0.01	<<0.01

* See Table III, footnote

Cats V, VIII, and IX. In the case of Cat V it was possible to obtain four assays of mitochondria prepared from hepatic biopsies, *viz.* diabetic, treated, relapsed, diabetic, treated. The rest of the data in Tables III and IV was obtained from depurinated cats either in the diabetic or in the normal state after insulin treatment. Table IV gives the values obtained: the means ± the standard error of the means, and the differences of the means between the normal and the diabetic status. The *P* values for these differences are highly significant, indicating again that there is an impaired phosphorylative capacity of the mitochondria from diabetic cat livers and more significantly that this depressed function is restored to normal by administration of insulin *in vivo*. This is emphasized further

by the statistical comparison of data from the normal cats (Table II) with that from depancreatized cats (Table IV) restored to a normal status by insulin treatment. This comparison shows no significant difference between the two.

We have never been able to demonstrate any effect of insulin added *in vitro* to mitochondrial preparations either from the normal or the diabetic cat on their oxidative phosphorylation assayed as described. On the other hand, our data suggest strongly that the presence within the intact animal of either endogenous or exogenous insulin maintains the mitochondria of the liver of the cat in such a state that, when isolated and assayed for phosphorylative capacity with pyruvate as a substrate, the mitochondria show high values of this capacity which may be termed characteristic of the normal state. In the diabetic cat, as our data show, phosphorylation by the isolated mitochondria falls to very low values, but normal values are again restored upon appropriate insulin treatment.

Livers of depancreatized cats are markedly fatty. Whether this is a cause or an effect of the decreased hepatic oxidative phosphorylation is undetermined. Dianzani and Scuro (11) subjected rats to prolonged treatment with a series of substances (*e g* 2,4-dinitrophenol) which produced a high degree of inhibition of oxidative phosphorylation by the liver both *in vitro* and *in vivo*. In these animals they observed an accumulation of fat within the liver cells. They concluded that "this fact favors the hypothesis that uncoupling of oxidative phosphorylation is an important step in the pathogenesis of fatty liver degeneration." The possibility that any hepatotoxin such as 2,4-dinitrophenol which produces fatty infiltration of the liver may non-specifically depress other metabolic functions, including oxidative phosphorylation, cannot be excluded by these experiments. We have sought to obtain some direct evidence on the possible role of fatty infiltration of the liver on the oxidative phosphorylation capacity of mitochondria derived from such livers. Houssay cats have fatty livers comparable to those of depancreatized cats. In mitochondrial preparations from two such cats, we observed normal phosphorylation values in one and normal P/O ratios in both. These preliminary observations would indicate that fatty infiltration *per se* does not depress hepatic oxidative phosphorylation.

As already mentioned, both the results of Parks *et al* (6) and our own studies on mitochondria from diabetic rat livers showed no departure from normal. We have no explanation for this species difference in mitochondrial response to insulin deprivation. The possibility that incomplete removal of insulin in the alloxan-diabetic rat is responsible for the difference must be considered.

Our data on the depressed oxidative phosphorylation of the isolated

mitochondria from diabetic cat livers are in accord with much evidence in the literature (8) that this impairment exists when the mitochondria are in place within the diabetic liver and contributes to certain metabolic defects in the diabetic status. In other words, the presence of insulin *in vivo* by some undetermined mechanism is required for the full maintenance of mitochondrial function. But in view of the fragile nature of the mitochondria and the possibility of damage during isolation, particularly in sick animals, our data can be regarded only as preliminary evidence for this conclusion. Much more experimental work is required before definite conclusions can be drawn.

SUMMARY

1 Data are presented from studies of oxidative phosphorylation of cat liver mitochondria with pyruvate as the substrate. Normal, diabetic, and diabetic insulin-treated animals were studied.

2 The mitochondria from diabetic animals show significant and marked decreases in phosphorylation rate, oxygen uptake, and P/O ratio. These changes were restored to normal by insulin treatment of the animal.

3 The significance of these findings is discussed.

We gratefully acknowledge the assistance of Dr. John F. Buse, Dr. Maria Gordon, and Dr. F. D. W. Lukens of the George S. Cox Institute, University of Pennsylvania, without whose cooperation in and supervision of many phases of the animal work this study could not have been completed.

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CONTROL BY URACIL OF FORMATION OF ENZYMES REQUIRED FOR OROTATE SYNTHESIS*

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Certain enzymes (constitutive) are found in bacteria at concentrations relatively independent of the nutrients present in the medium (1, 2). In contrast, other enzymes (inducible) are dependent for their formation on the presence of specific compounds, usually their substrates (3). What mechanisms are used by the bacteria to maintain their constitutive enzymes at fixed concentrations? Precise control mechanisms are indicated by the remarkable coordination, during growth, of the syntheses of essential bacterial constituents such as amino acids, purines, and pyrimidines (4). Presumably, the rates of reactions catalyzed by key enzymes control the syntheses of these small molecules, and regulation of these rates can be achieved either by specific influences of the intracellular small molecules on the *activity* of the enzymes (5) or, alternatively, by regulation of the *amounts* of the various enzymes. It has been suggested that some constitutive enzymes are under nuclear control, their formation is not specifically influenced by the small molecules in the cytoplasm (6). But substrates and products of the essential reactions catalyzed by these constitutive enzymes are normally present in the bacteria, and these compounds could mediate the formation of the enzymes (7). Few data have been accumulated in support of these hypotheses (see "Discussion").

An approach to these problems was found in certain observations on the activities of three enzymes required for orotic acid synthesis (ureido-succinic synthetase, dihydroorotase, and dihydroorotic dehydrogenase). The activities of these enzymes in *Escherichia coli* were found to be of approximately the proper magnitude to provide an adequate supply of pyrimidines to the growing bacteria, and, since the enzymes were found in cells grown in a salts-glycerol medium (8), they can be classified as constitutive. In another organism, *Zymobacterium oroticum*, two of these enzymes reached much higher levels when the bacteria were grown on orotate than when they were grown on glucose as the sole carbon source, therefore, the enzymes, though still essential, were also inducible. It

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seemed possible that in *E. coli*, too, the amounts of the enzymes could be made to vary, and experiments were performed to accomplish this. It was soon found that activities were easily altered and were dependent on the intracellular concentrations of metabolites which were not substrates of the enzymes, but rather end products of the metabolic pathway. These metabolites acted as inhibitors of enzyme formation.

Materials and Methods

L-Aspartic acid was obtained from the Nutritional Biochemicals Corporation, uracil, OA,¹ and other pyrimidines were obtained from the California Foundation for Biochemical Research, and 6-azauracil was a gift from Dr. E. Sassenrath and was prepared by Dr. A. D. Welch. DL-CAA, L-DHO, and CP were prepared in these laboratories as described previously (8). 5-Methyl-DL-tryptophan was obtained from the H. M. Chemical Company. Chloromycetin was a gift from Parke, Davis and Company.

The bacteria used in these experiments were *E. coli*, strain B, and some pyrimidine-requiring mutants of other strains of *E. coli*. Mutant R 185-482, isolated by Dr. R. R. Roepke, was kindly provided by Dr. B. D. Davis. This mutant requires citrulline or arginine plus uracil, cytosine, CAA, DHO, or OA for growth, and apparently lacks the ability to form CP. Mutant 6386 (ATCC No. 12632), also obtained from Dr. B. D. Davis, had earlier been found to lack the enzyme dihydroorotic acid dehydrogenase (8). Mutant 58-5417, kindly provided by Dr. S. R. Gross, had been characterized in this publication as lacking the enzyme dihydroorotase. Mutant 550-460 (ATCC No. 11548), isolated by Dr. R. R. Roepke, grows on uracil or uridine and accumulates CAA, DHO, and OA. It therefore is blocked somewhere in the conversion of OA to uridine 5'-phosphate.

The bacteria were grown at 37° in liquid culture, being aerated by swirling. The minimal medium consisted of inorganic salts plus glycerol as an energy source (8). If not stated elsewhere, 4×10^{-4} M arginine and 2×10^{-4} M uracil, OA, DHO, or CAA were used when needed to permit growth.

Cell-free extracts were prepared by subjecting bacteria to sonic oscillation in a 9 kc Raytheon magnetostriction oscillator at 4° for 10 minutes. Bacteria in samples of less than 5 ml. could be broken by placing them in a plastic centrifuge tube which was floated in 15 ml. of water in the oscillator cup. The extracts were centrifuged 3 minutes at $8000 \times g$ to remove whole cells and cellular debris.

¹ The abbreviations used in this paper are carbamyl aspartic acid (ureidosuccinic acid), CAA, dihydroorotic acid, DHO, orotic acid, OA, carbamyl phosphate, CP, ribose nucleic acid, RNA.

Of the enzymes tested, *D-serine deaminase* and β -galactosidase were assayed as described previously (9, 10), and the other enzymes as described below. *Aspartate carbamyl transferase* (11) (ureidosuccinic synthetase)-reaction mixtures contained 0.05 M potassium phosphate buffer (pH 7.0), 0.015 M L-aspartic acid, 0.008 M CP, and extract capable of forming about 2 μ moles of CAA per ml of reaction mixture. The 2.0 ml reaction mixture was incubated 20 or 60 minutes at 25°, depending upon enzyme activity. The reaction was halted by rapid passage of a 1 ml aliquot through a 1 ml Dowex 50 (acid form) column (in order to remove citrulline), followed by an equal volume of water to rinse the column. The eluate was assayed for CAA (12). Occasionally assays for DHO and OA were performed, but these two compounds together rarely amounted to 10 per cent of the CAA value. In some experiments CAA was determined by the method of Knivett (13), in which case it was not necessary to pass the assay mixture through a column, and the reaction was halted with 4 per cent HClO_4 .

Dihydroorotase—Reaction mixtures contained 0.1 M sodium acetate buffer (pH 5.5), 0.008 M DL-CAA, and an extract of about 6×10^9 bacteria (equal to about 0.9 mg of protein) in 3.0 ml. The reaction mixture was incubated at 25°, and samples taken at 10 and 20 minutes were run through ion exchange columns as above and assayed for DHO (8). Neither aspartate carbamyl transferase nor DHO dehydrogenase is active at the pH of this assay.

Dihydroorotic Acid Dehydrogenase—Reaction mixtures contained 0.05 M potassium phosphate buffer (pH 7.5), 0.002 M DHO, and 0.45 mg of extract protein in 3.0 ml. The control sample lacked DHO. The optical density of the reaction mixture, in a silica cuvette, was measured at 290 m μ with a Beckman model DU spectrophotometer by using the control sample as a blank. Readings were made at 30 second intervals for 6 minutes, and the initial rate of production of OA was calculated ($\epsilon_{\text{OA}} = 6.2 \times 10^3$) (8). Protein measurements were made by the Folin-Ciocalteu method (14), and ribonucleic acid was determined by the Mejbaum orcinol method (15).

Starch Electrophoresis—Cell-free extracts were fractionated with the starch electrophoresis apparatus described by Paigen (16). An extract of about 6×10^{10} bacteria was placed near one end of a tray 30 cm long, 3 cm wide, and containing 40 gm of starch and 35 ml of 0.02 M tris-(hydroxymethyl)aminomethane buffer, pH 7.6. 150 volts were applied for 15 hours. The starch trays were cut in 0.5 cm sections and eluted with water, and the eluates were assayed for the desired substances.

Ultraviolet Irradiation—Heavy irradiation of bacterial suspensions in minimal salt solutions, 0.5 cm deep, was given by a 2 minute exposure to three Westinghouse sterile lamps, No. WL-782L-30, at a distance of 14

inches After this treatment, formation of nucleic acids by the bacteria was essentially abolished Mild irradiation was given by a 0.35 ampere Rad-i-air lamp with an intensity of $350 \text{ ergs sec}^{-1} \text{ cm}^{-2}$ at 85 cm

EXPERIMENTAL

Stimulation of Enzyme Production by Pyrimidine Depletion—The three enzymes of orotic acid synthesis studied here were present in *E. coli*, strain B, grown on a salts-glycerol medium, and hence they appeared to be typical constitutive enzymes (8) When attempts were made to bring

TABLE I
Effects of Uracil on Enzyme Formation

<i>E. coli</i>	Uracil	Specific activity		
		ACTase	DHOase	DHODEh
B	—	0.47	0.69	0.49
58-5417	+	0.34	0.00	0.35
	—	88.0	0.00	3.4
6386	+	0.40	1.0	0.00
	—	86.5	8.7	0.00
550-460	+	0.37	0.07	0.34
	—	48.6	3.0	1.31

The bacteria were grown to the exponential phase in salts-glycerol medium supplemented with uracil in the case of the mutants They were centrifuged and resuspended in fresh medium with or without uracil, and aliquots were taken for enzyme and protein assays at 110 minutes The enzyme activities are given as micro moles per mg of protein per hour The abbreviations are ACTase for aspartate carbamyl transferase, DHOase for dihydroorotase, and DHODEh for dihydroorotic dehydrogenase

about variations in the activities of these enzymes in *E. coli*, it was found that all three of the enzymes showed great increases in specific activity when the bacteria were deprived of pyrimidines Pyrimidine depletion was attained in pyrimidine-requiring mutants, grown in the presence of uracil, by washing the bacteria and aerating them at 37° in the minimal medium Each of the mutants initially had enzyme activities similar to those of the wild type (*E. coli*, strain B), but after pyrimidine starvation the activity of aspartate carbamyl transferase had increased 150- to 500-fold, dihydroorotase 10- to 40-fold, and dihydroorotic dehydrogenase 4- to 10-fold (Table I)

The absence of a pyrimidine supply did not result in an accelerated formation of cellular components in general, in fact, quite the opposite result was observed (Fig. 1) After the pyrimidine-requiring mutant had

taken up all available uracil, as indicated by abrupt cessation of RNA synthesis and the appearance of OA in the medium (8) at 35 minutes, the synthesis of protein and of the inducible enzyme D-serine deaminase was halted within 10 minutes. These observations are in agreement with, and

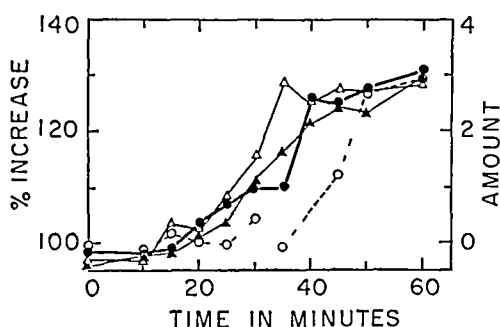


FIG 1 Formation of various materials upon depletion of uracil. Mutant 550 460 grown on salts-glycerol medium plus uracil was centrifuged and resuspended in a similar medium containing 1 γ per ml of uracil. Subsequently, assays were made for various materials. The results for protein (●) and RNA (△) are expressed as per cent changes over the initial values, orotic acid (○) and D-serine deaminase (▲) are increases over initial values in arbitrary units.

TABLE II
Effects of Rich Medium on Enzyme Formation

Supplements	Specific activity		
	ACTase	DHOase	DHOdeh
Arginine + uracil	0.31	0.15	0.37
" + DHO	150.0	1.07	3.60
1% broth and 0.1% yeast extract	4.7	0.29	0.71
1% " 0.1% yeast extract, and DHO	3.3	0.15	0.66

E. coli mutant R 185-482 was grown on salts-glycerol medium plus arginine and uracil, and was centrifuged, resuspended, and aerated for 110 minutes in media with the above supplements. Assays were made for enzymes and protein. The units and abbreviations are described in Table I.

extend, prior observations on the dependence of β -galactosidase formation on a supply of pyrimidines (10, 17).

The relation between pyrimidine starvation and enhanced activity of the enzymes of orotic acid synthesis is also specific in the sense that other methods of upsetting the bacterial metabolic economy did not affect these enzyme activities nearly as greatly. In one set of experiments, a rich medium was used (Table II), mutant R 185-482 grown in this medium had

a 10-fold enhanced aspartate carbamyl transferase activity, and dihydroorotase and dihydroorotic dehydrogenase were increased 2-fold each over the values found in the same strain grown in minimal medium plus uracil and arginine

The effects of a less adequate medium were also tested, growth of mutant 6386 in a minimal medium plus uracil was inhibited by addition of 5-methyl-DL-tryptophan. This inhibitor presumably acts by preventing the synthesis of tryptophan (18). At a concentration of 2 γ per ml it permitted only a slight protein increase, while the specific activity of aspartate carbamyl transferase usually increased by a factor of only 2. The actual results were quite variable, however. The experiment shows that this method of slowing growth did not stimulate formation of these enzymes.

To determine whether mild ultraviolet irradiation specifically affects the ability of *E. coli* to form these enzymes, a rapidly growing culture of mutant R 185-482 was washed and suspended in minimal medium without a carbon source, and aliquots were irradiated with ultraviolet light for various lengths of time. The bacteria, kept in the dark to avoid photo-reactivation, were then diluted into minimal medium containing arginine and DHO and incubated at 37°. At various times after irradiation, suitably diluted aliquots were placed on broth (plus uracil) agar plates for viable counts, and other aliquots were assayed for enzyme activities. Irradiation reduced the viable count to as little as 3 per cent of the control, and formation of each of the three enzymes was depressed by a factor of 2 or less. There was no difference in ability to synthesize protein in the irradiated and non-irradiated cultures. Evidently, any imbalances created by lethal doses of irradiation were not capable of preferentially bringing about increased enzyme activities.

The results of kinetic studies of enzyme formation after removal of uracil are shown in Fig. 2. Formation of the three enzymes took place at a far greater rate in the absence than in the presence of uracil. There was no lag in the formation of aspartate carbamyl transferase or DHO dehydrogenase, and in fact it was difficult to obtain their true initial activities. A lag was observed in the formation of dihydroorotase, but this enzyme is unstable and the first enzyme molecules formed may have been inactivated, *e.g.* by traces of heavy metal ions. A pyrimidine precursor (CAA or DHO) was required for enzyme formation, but only after about 30 minutes. It is likely that traces of intracellular pyrimidines were adequate initially to allow protein formation. Little increase in protein was observed in these experiments in either the presence or the absence of DHO, the protein increased only by about 20 per cent in 2 hours, whereas the control increased 300 per cent.

The effect of resumption of growth is shown in Fig 3 In this experiment, after enzyme formation in minimal medium (plus arginine), the bacteria were provided with fresh medium containing uracil and arginine Rapid growth commenced, but specific activities of the enzymes decreased Formation of these enzymes was then not as rapid as total protein synthesis,

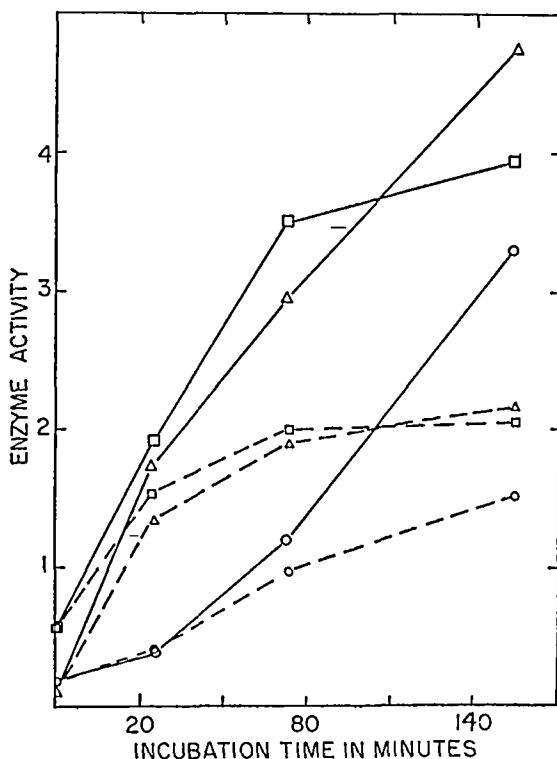


FIG 2 Specific activity changes in mutant R 185-482 after deprivation of uracil The experiment was performed as for Table I Aliquots were taken at intervals for assay (Δ) Aspartate carbamyl transferase, (O) dihydroorotase, (□) dihydroorotic dehydrogenase Solid lines were for cultures in the presence of DHO and dashed lines for those obtained in its absence Activities are in arbitrary units per mg of protein

however, the formation of the enzymes did not cease abruptly but continued less rapidly for an hour or more The enzymes were stable under growth conditions, but the conditions for their formation were not preserved

Compounds Which Control Formation of Enzymes of Orotic Acid Synthesis— The marked increases in enzyme activities, described above, are suggestive of the specific increases brought about in other cases by addition of low molecular weight compounds to the culture medium (enzyme induction)

Experiments were therefore directed toward determining whether the changes in activity found above could be attributed to changes in the concentrations of some small molecular weight compounds. Three hypotheses as to the mechanism of control of synthesis may be made: (1) CAA or another pyrimidine intermediate is required as an enzyme inducer and is present at high concentration only in non-growing bacteria; (2) Uracil, or some derivative thereof, present at moderate or low concentrations in growing bacteria but absent in resting uracilless mutants, inhibits enzyme formation; (3) Some balance of metabolites other than

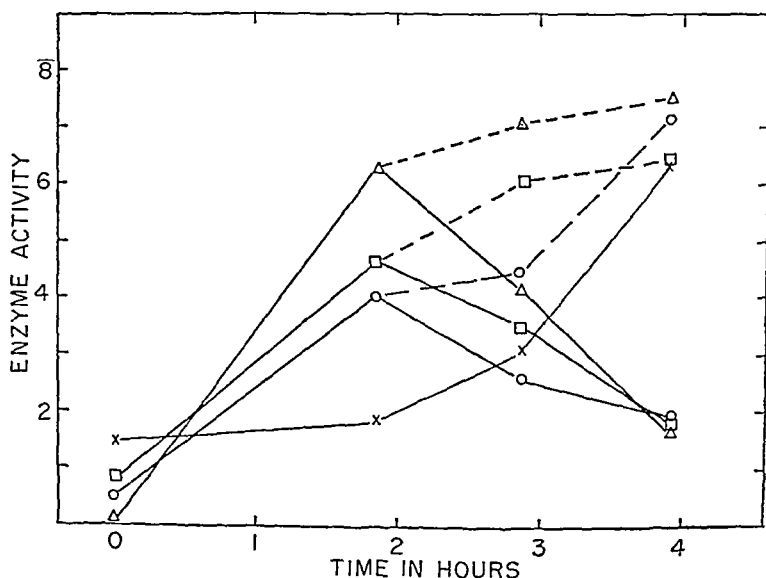


FIG 3 Recovery from uracil deprivation. The experiment was performed like that described for Fig 2, except that at 2 hours uracil was added. Specific activities are shown as solid lines, and activities per ml are plotted as dashed lines. Symbols are the same as those used for Fig 2. Protein values are shown by (X).

pyrimidines, different in rapidly growing bacteria as compared to bacteria under conditions of pyrimidine starvation, is responsible, for example, the systems which form the three orotic acid-synthesizing enzymes might not be able to compete successfully with other enzyme-forming systems for metabolites such as amino acids, except that the former enzymes might be made preferentially if formation of other enzymes is inhibited by a low supply of pyrimidines.

The first mechanism, typical of enzyme induction, is ruled out by two kinds of evidence. Mutant R 185-482 appears to lack the ability to form CP and does not accumulate CAA, DHO, or OA in the medium. Growth of this mutant with uracil should result in virtual absence of these pyrimidine precursors in the bacteria, especially since the metabolic sequence is

irreversible at the step between orotidylc and uridylic acids (19). Therefore, if CAA, DHO, or OA were required as inducers of any of the three enzymes tested, these enzymes should be formed in lower concentrations in this mutant, under all conditions, than in the wild type bacteria. This was not the case. Similarly, marked increases of enzyme activities were found for three other mutants (Table I). In minimal medium, mutant 58-5417 produced only the precursor CAA, mutant 6386 produced CAA and DHO, and mutant 550-460 produced CAA, DHO, and OA, yet all the mutants formed similar amounts of each of the three enzymes. There-

TABLE III
Comparison of Effects of Uracil and Orotate

Experiment No	<i>E. coli</i>	Pyrimidine supplement	Per cent protein increase	Specific activity			
				ACTase	DHOase	DHODEH	β Gal
1	R 185-482	Uracil	232	0.31	0.15	0.37	286 190
		OA	223	0.70		0.36	
2	6386	" + uracil	105	0.2			
		"	72	71.0			
3	6386	Uracil	255	0.4	1.0	0.00	
		OA	242	11.5	1.9	0.00	
		None	21	86.0	8.6		

The experiments were performed as in Table I. In Experiment 1, mutant R 185-482, which grew as well on OA as on uracil, was incubated for 110 minutes. The strain of mutant 6386 used for Experiment 2 grew relatively slowly on OA. It was exposed for 60 minutes in the presence of 1 mg per ml of lactose as an inducer for β -galactosidase (β -Gal). In Experiment 3 a substrain of mutant 6386 that grew rapidly on OA was incubated for 110 minutes.

fore, the concentrations of pyrimidine precursors required for formation of these enzymes must be extremely small, if such precursors are required at all.

The inability of pyrimidine precursors to act as inducers was shown in yet another way, *ie.*, by demonstration of absence of induction in the presence of substrates of the enzymes. Substrains of mutant R 185-482 were selected which grew rapidly on CAA, DHO, or OA. Cultures grown with each of these compounds as the sole pyrimidine precursor had the same low activity of the three enzymes as a culture grown with uracil, even though precursors were present in high concentrations (Table III). Similarly, when the mutant was grown on a rich medium, addition of DHO had no influence on the enzyme levels (Table II). By contrast,

strains of the same mutant which grew slowly on CAA or DHO showed greatly increased enzyme activities in the presence of these compounds (Fig 2) It is likely that in the latter case the precursors served to provide a continuous minute supply of pyrimidines Presumably, slow growth was due to low permeability since enzymes for metabolism of CAA to OA were present, and OA permitted rapid growth

The second mechanism of control requires that formation of the enzymes be inhibited whenever surplus pyrimidines are present in the bacteria, and the third mechanism requires inhibition by a suboptimal supply of some nutrient other than pyrimidines It is difficult to distinguish between these possibilities A suitable method might be to study enzyme activities under conditions whereby pyrimidines are removed as rapidly as they become available but which permit nearly normal rapid growth, so that other nutrients are at concentrations similar to those found in cultures possessing surplus pyrimidines This objective was accomplished in three ways, and in each case a marked stimulation of aspartate carbamyl transferase activity was observed First, it was noted that substrains of mutant 6386 grew slightly less rapidly with OA than with uracil, hence the pyrimidine supply limited growth in the former case Measurements of aspartate carbamyl transferase showed far greater activities in bacteria grown on OA than in those grown on uracil (Table III) Bacteria grown in the presence of both OA and uracil had low activities, therefore the differences were due to an inhibition by uracil rather than to a stimulation by OA Other data (Table III) show that β -galactosidase formation varied in the same way as did protein, so that other nutrients were probably in similar supply under both conditions

A second method of limiting the supply of pyrimidines was by addition of 6-azauracil (20) to the medium This compound prevents formation and utilization of OA by *E coli*,² when it is added at low concentrations it limits growth by creating a pyrimidine deficiency The effects of its presence on formation of protein, β -galactosidase, and aspartate carbamyl transferase by *E coli* strain B and the mutant 6386 are seen in Table IV Azauracil has been shown to inhibit β -galactosidase formation³ Growth and β -galactosidase formation were only slightly inhibited, and yet the transferase activity was greatly stimulated These results strongly implicate intrabacterial pyrimidines as inhibitors of formation of this enzyme

Table IV also indicates that higher concentrations of 6-azauracil inhibited formation of aspartate carbamyl transferase This action appears to be a specific inhibition rather than part of the generalized inhibition of all syntheses owing to a low supply of pyrimidines, because protein and

² Pardee, A B , and Prestidge, L S , unpublished

³ Personal communication, Handschumacher, R E

β -galactosidase data obtained with *E. coli* strain B showed that pyrimidine deficiency was not severe in the presence of 6-azauracil. This observation

TABLE IV
Effects of 6-Azauracil

Strain	Time	Pyrimidine supplement		Protein mg per ml	Specific activity	
			γ per ml		ACTase	β Gal
B	30	None		0.325	1.35	288
		Uridyl	20	0.340	1.45	276
		Azauracil	20	0.320	30.0	248
		"	10	0.350	19.6	200
6386	115	None		0.205	138	5.0
		OA	20	0.415	67	15.0
		" + azauracil	20	0.275	106.0	8.0
		" + "	100	0.210	37.0	5.0

This experiment was performed in the same manner as that described in Table I but with the strains shown in the first column. Lactose, 1 mg per ml, was added as an inducer for β -galactosidase (β Gal).

TABLE V
Short Time Effects of Transfer to Rich Medium

Supplements	Protein	Specific activity	
		ACTase	β Gal per ml
	mg per ml		
None	0.325	1.35	14.0
10 γ + adenine	0.415	7.2	7.0
10 γ + " + uracil	0.400	2.2	7.0

E. coli strain B were grown to the exponential phase on salts-glycerol medium, and aliquots were transferred to media containing 1 mg per ml of hydrolyzed casein plus 10 γ per ml of tryptophan (10) and 10 γ per ml of adenine, without and with 20 γ per ml of uracil. Lactose (1 mg per ml) was added as an inducer for β -galactosidase. Aliquots were removed at 15 minutes for β -galactosidase and at 30 minutes for aspartate carbamyl transferase and protein.

supports the idea that pyrimidine derivatives inhibit transferase formation.

A third method of creating a pyrimidine deficiency is to transfer the bacteria to a rich medium, i.e., one containing as many nutrients in excess, other than pyrimidines as possible (17). Such a change of medium (see Table V) brought about a considerable increase in transferase activity,

and, at the same time, a considerable inhibition of β -galactosidase formation resulted for a short period. The simultaneous addition of uracil inhibited the transferase formation considerably, as would be expected from the second hypothesis of the method of control. However, β -galactosidase synthesis was not restored, thus, nutrients other than uracil must have been in short supply also. These three observations favor the hypothesis that the formation of the enzymes is controlled by pyrimidines rather than by availability of trace nutrients, inasmuch as the enzymes

TABLE VI
Effects of Inhibition of Protein Synthesis

<i>E. coli</i>	Inhibitor	Supplements	Specific activity		
			ACTase	DHOase	DHOdeh
R 185-482	None	DHO, arginine	150	1 07	3 6
	No arginine	None	2 4	0 39	0 32
	Heavy ultraviolet	DHO, arginine	1 3	0 41	0 29
6386	None	OA	71		
	5-Methyltryptophan	"	0 7		
	Chloromycetin	"	0 3		

In the first experiment, mutant R 185-482 was grown in the presence of arginine and uracil, centrifuged, and resuspended in new media containing the supplements and inhibitors shown above. One aliquot was treated with strong ultraviolet light. Cultures were aerated for 110 minutes and then analyzed. The second experiment was similar except that mutant 6386 was employed, 40 γ per ml. of 5-methyltryptophan or Chloromycetin were added to the cultures, and exposure was for 60 minutes. At the end of each experiment, assays were made for protein and enzymes. The results are expressed as in Table I.

are formed under faster and slower growth rates, but only when the pyrimidine supply is low.

Role of Protein Synthesis—It may be asked whether the great increases of enzyme activities described above result from formation of new enzyme molecules or are brought about by some process of activation of preexisting enzymes. This question is especially pertinent because the activities increased most under conditions whereby other enzymes were not rapidly made. It was first determined that the enzymes of orotic acid synthesis could not become more active under various conditions of inhibited protein synthesis (Table VI). The increased activities of these enzymes required the presence of arginine, and were inhibited by Chloromycetin, 5-methyltryptophan, or a heavy dose of ultraviolet light. It will be recalled that a requirement for traces of pyrimidines exists (Fig. 2).

A more direct demonstration of *de novo* formation of aspartate carbamyl transferase was provided by an experiment in which it was shown that if extracts of pyrimidine-starved bacteria were distributed in the starch electrophoresis apparatus, much newly formed protein moved with the aspartate carbamyl transferase fraction. For this study, a growing culture of mutant R 185-482 was washed, resuspended in minimal medium plus arginine, and permitted to respire in the presence of C^{14} -leucine for 60 minutes (all leucine should have been used within 5 minutes). A cell-free extract was prepared by sonic oscillation and partly resolved by

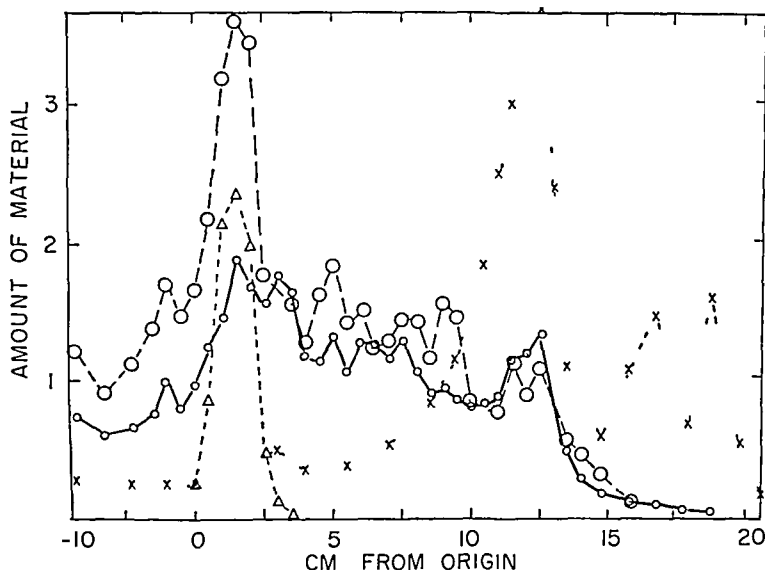


FIG. 4 Starch electrophoresis of an extract of mutant R 185 482 to which leucine- C^{14} was added after the removal of uracil (X) optical density at 260 $m\mu$ (nucleic acids), (O) protein, (O) C^{14} , (Δ) aspartate carbamyl transferase. Ordinates are in arbitrary units.

starch electrophoresis. The patterns of protein, C^{14} , aspartate carbamyl transferase, and optical density at 260 $m\mu$ (as a measure of nucleic acids) are presented in Fig. 4. It is seen that C^{14} was distributed in a manner similar to total protein except at the position where the enzyme was found. Calculations from these data show that several per cent of the newly formed (C^{14}) protein may be aspartate carbamyl transferase.

As a control, a rapidly growing culture of mutant R 185-482 was exposed to C^{14} -leucine, and an extract was prepared and treated as above. The patterns of protein and C^{14} corresponded at all points, within experimental error, unlike the results in Fig. 4. Therefore these results show that the increased transferase activity is accompanied by selective synthesis of a new protein of mobility similar to the transferase.

These results indicate that enough aspartate carbamyl transferase is present (perhaps 1 per cent of the total protein) to make its purification from pyrimidine-starved bacteria feasible. Preliminary experiments have resulted in a 50-fold purification by conventional procedures of ammonium sulfate fractionation at pH 7.0 (39 to 50 per cent saturation), heat inactivation (65°), and isoelectric precipitation (pH 5.5), followed by starch electrophoresis. The highest specific activity obtained was 3.5 moles of CAA formed per hour per gm of protein. This activity is considerably greater than that obtained by Reichard and Hanshoff (11), as would be expected from the relative activities of the starting materials.

DISCUSSION

Three enzymes of orotic acid biosynthesis in *E. coli* were shown to be capable of great variations of activities, depending on conditions of culture of the bacteria. Furthermore, the increases observed represent actual formation of enzyme proteins and not simply activation of preexisting enzymes. The question arises as to how these potentialities for enzyme formation are held at a relatively constant low level, one convenient for supplying the growth requirements of the bacteria without waste under most conditions. In other words, why do the enzymes normally act like constitutive enzymes? The question is answered by the discovery that formations of these enzymes are under the control of end products of their metabolic pathway (clearly proved at least in the case of aspartate carbamyl transferase). It has not been possible to determine what compounds actually function as "repressors" (21) of enzyme formation, but they must be readily derived from uracil, a potent inhibitor, or from 6-azauracil (which is similarly anabolized).³

This method of control, by enzyme repression, should be distinguished from other types of control. It has previously been shown that a pyrimidine derivative, probably cytidine, controls the rate of pyrimidine biosynthesis by inhibiting the *activity* of aspartate carbamyl transferase (5). This mode of control is clearly distinct from the control of enzyme *synthesis* described above; it seems reasonable to suppose that both regulatory mechanisms are of use to *E. coli*. Enzyme induction (specific control of enzyme formation by the substrate) must also be distinguished from the above two regulatory mechanisms. (The fourth member of this set of regulatory mechanisms is the most commonly described, *i.e.* enzyme activities are dependent on substrate concentrations.) The complementary modes of action on enzyme formation of repressors and inducers have implications regarding the mechanism of enzyme formation (21) which will not be discussed here. It is worth mentioning that the enzymes are maintained at a basal level even when the bacteria are grown in the presence of uracil.

Such a powerful mode of metabolic regulation as enzyme repression could be of primary importance in the growth of unicellular organisms (22). That some such mechanism may be widespread has been suggested recently by Neidhardt and Magasanik (23) in view of experiments on the inhibition by glucose of synthesis of some inducible enzymes. A number of observations, both general (4) and specific (7, 24, 25), support the view that repression mechanisms are widespread. The most complete demonstration of repression is of acetylornithinase formation by arginine, shown very recently by Vogel (21).⁴ The entire problem is made difficult in that it is hard to find "gratuitous conditions" (3). Shortages in the amounts of small molecules required for essential biosyntheses have profound non-specific effects on enzyme formation, and these effects interfere with observation of their possible specific effects.

Both in the present case and in the preferential formation of β -galactosidase in response to addition of galactosides (26, 27), enzymes are formed, the action of which normally helps to overcome the metabolic deficiency. Any such mechanism would evidently be most useful to the bacteria under conditions of remediable stress.

Finally, it seems clear that, while the term "constitutive enzyme" (2) has meaning at the nutritional level of investigation, it is not at present useful at the level where interactions of molecules are considered.

SUMMARY

1 It was possible to cause three "constitutive" enzymes required for orotic acid formation in *Escherichia coli* to increase in their specific activities by one to two orders of magnitude.

2 The formation of these enzymes is controlled by a mechanism of enzyme repression. End products of the metabolic pathway, derived from uracil, inhibit enzyme formation.

3 Inhibition studies and experiments with radioactive leucine incorporation show that actual enzyme formation is involved, rather than enzyme activation.

4 The above results are discussed in relation to other modes of metabolic control.

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⁴ We are indebted to Dr. Vogel for a copy of his article prior to publication.

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REDUCTIVE DEGRADATION OF PYRIMIDINES

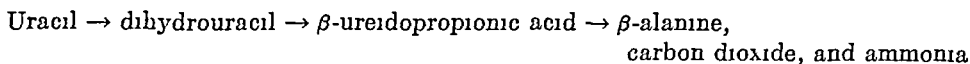
III PURIFICATION AND PROPERTIES OF DIHYDROURACIL DEHYDROGENASE*

By L. LEON CAMPBELL, Jr

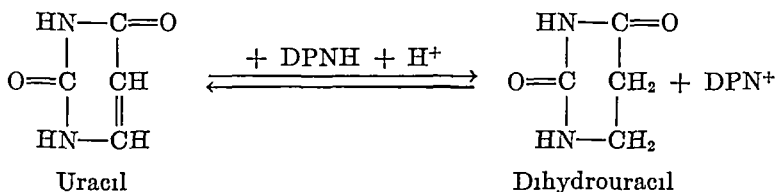
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(Received for publication, November 21, 1956)

In Papers I and II in this series, evidence was presented for the reductive pathway of uracil degradation by *Clostridium uracilicum* (1, 2). This pathway may be viewed as follows:



The purpose of this paper is to describe the purification and properties of the enzyme, dihydrouracil dehydrogenase, and to present further evidence for the following reaction:



EXPERIMENTAL

Methods—*C. uracilicum*, strain M5-2, was used, and the methods of culture and preparation of cell-free extracts were described previously (2).

Glucose dehydrogenase was purified as described by Strecker and Korkes (3), calcium phosphate gel was prepared according to Keilin and Hartree (4). Glucose was estimated spectrophotometrically with hexokinase (5), and glucose-6-phosphate dehydrogenase (6) as described by Lieberman and Kornberg (7). Uracil was determined by optical density measurement at 260 mμ in a Beckman DU spectrophotometer, and protein by the method of Lowry *et al.* (8). Paper chromatography of reaction mixtures was carried out as described previously (2).

Assay of Dihydrouracil Dehydrogenase—It has been established that reduced diphosphopyridine nucleotide (DPNH) is required for the reduction of uracil to dihydrouracil (2). For assay purposes DPNH was gen-

* Scientific Paper No. 1520, Agricultural Experiment Stations, Institute of Agricultural Sciences, State College of Washington, Pullman, Washington.

erated by the addition of glucose, glucose dehydrogenase, and diphosphopyridine nucleotide (DPN) to the test system. The reaction mixture contained 10 μ moles of $MgCl_2$, 150 μ moles of potassium phosphate buffer, pH 7.4, 0.5 μ mole of uracil, 10 μ moles of cysteine, 0.05 μ mole of DPN, 200 μ moles of glucose, 250 units of glucose dehydrogenase, and the enzyme in a total volume of 3.0 ml. All the components except glucose dehydrogenase were incubated for 10 minutes at room temperature. Glucose dehydrogenase was added and the rate of uracil removal was determined by measuring the decrease in optical density at 260 $m\mu$. A unit of enzyme is defined as that amount which produces a reduction in optical density of 0.100 in 10 minutes; specific activity is expressed as units per mg of protein. In the absence of glucose, glucose dehydrogenase, or DPN, no removal of uracil was noted.

Purification of Dihydrouracil Dehydrogenase—All purification steps were carried out at 0–5°.

Removal of Nucleic Acid—Nucleic acid was removed from the cell-free extract by precipitation with manganese chloride as described by Korkes *et al.* (9). 25 ml of a 1.0 M solution of $MnCl_2$ were added to 500 ml of extract and stirred for 10 minutes. The stringy precipitate was removed by centrifugation and discarded. The ratio of optical densities at 280 and 260 $m\mu$ (10) was determined on the supernatant liquid, and it (1.40) indicated that essentially all of the nucleic acid was removed by this treatment. The supernatant solution was dialyzed for 12 hours against 0.05 per cent sodium sulfide.

Ammonium Sulfate Fraction—The dialyzed solution was adjusted to pH 6.0 with dilute acetic acid, and 185 gm of solid ammonium sulfate were added with stirring for 25 minutes, after which the precipitate was removed by centrifugation and discarded. Ammonium sulfate (38 gm) was added to the supernatant solution and stirred for 25 minutes. The precipitate was removed by centrifugation and dissolved in 100 ml of water.

Acid Ammonium Sulfate Fraction—To the ammonium sulfate fraction were added 25 ml of sodium acetate buffer (0.2 M, pH 4.6) and 30 gm of ammonium sulfate with stirring. After the fraction was stirred for 15 minutes, the precipitate was removed and discarded and to the supernatant solution were added 20 gm of ammonium sulfate. After the solution was stirred for 10 minutes, the precipitate was removed and dissolved in 40 ml of sodium acetate buffer (0.01 M, pH 6.0).

First Calcium Phosphate Gel Treatment—Calcium phosphate gel (0.2 volume) was stirred into the solution (pH 6.0). The suspension was stirred for 15 to 20 minutes and centrifuged, and the gel discarded. This step removed considerable inert protein and a minimum of enzyme.

Second Calcium Phosphate Gel Treatment—The solution was adjusted to pH 5.5, and 0.6 volume of calcium phosphate gel was added to adsorb the

enzyme completely After being stirred for 15 to 20 minutes, the gel was removed by centrifugation and washed three times with 5 volumes of water The wash water and the supernatant fluid were discarded The enzyme was eluted from the gel by stirring for 30 minutes with 0.2 M potassium phosphate buffer, pH 7.4 The volume of buffer used was approximately one-fourth of the volume of the solution after the first gel treatment

TABLE I
Purification of Dihydrouracil Dehydrogenase

Fraction	Volume of solution	Units	Specific activity	Yield
	<i>ml</i>		<i>units per mg</i>	<i>per cent</i>
Cell-free extract	500	35,000	16.66	
Dialyzed MnCl ₂ supernatant fluid	600	22,400	26.12	64
Ammonium sulfate	100	21,000	80.0	60
Acid ammonium sulfate	40	18,666	108.0	53
1st calcium phosphate gel treatment	44	14,000	252.0	40
2nd " " " "	10	9,000	450.0	26

TABLE II
Effect of Enzyme Concentration on Uracil Reduction

Amount of enzyme	Δ optical density
<i>ml</i>	<i>260 mμ</i>
0.00	0.000
0.02	-0.048
0.04	-0.108
0.06	-0.152
0.08	-0.200
0.10	-0.260

The conditions were as described in the text for routine assay

Data on the various steps in a typical procedure (Table I) show a 27-fold purification of the enzyme This is in the range of purification usually obtained (20- to 30-fold) by the fractionation procedure employed

Specificity of Purified Enzyme—The enzyme was tested for activity with cytosine, 5-methylcytosine, thymine, and orotic acid No activity was obtained with any of these compounds Furthermore, the enzyme does not convert dihydrouracil, β -ureidopropionic acid, or carbamyl phosphate to end products

Influence of Enzyme Concentration on Rate of Uracil Reduction—Under the conditions of the routine assay the rate of uracil reduction was proportional to the amount of enzyme present Typical data are given in Table II

These values are in essential agreement with those reported for dihydrouracil by Batt *et al* (12) Paper chromatography of the alkaline decom-

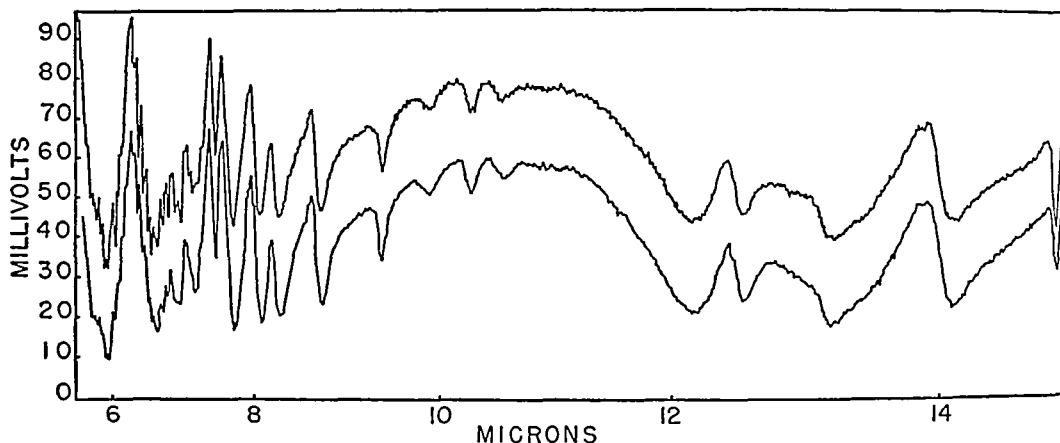


FIG 2 Infrared spectra of authentic dihydrouracil (upper curve) and the enzymatic product (lower curve) in KBr on a Perkin-Elmer recording spectrophotometer. The peaks at 13.88 and 15.0 μ are due to CO₂ absorption.

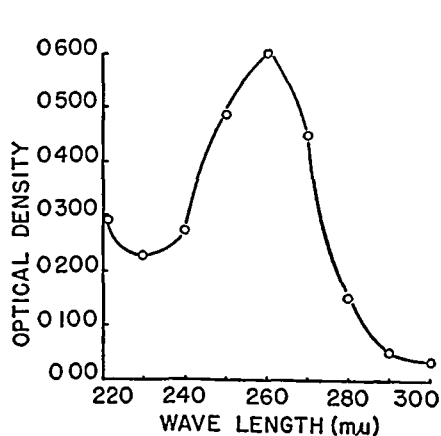


FIG 3

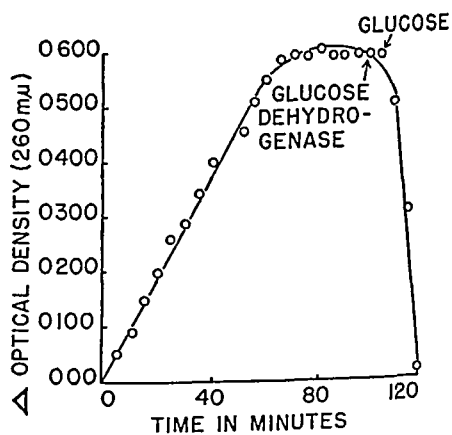


FIG 4

FIG 3 Absorption spectra of uracil and the product of dihydrouracil oxidation. The curve represents the spectrum of uracil. O, values obtained upon complete oxidation of dihydrouracil under the conditions described in Fig 4.

FIG 4 The oxidation of dihydrouracil to uracil. The reaction mixture contained MgCl₂, 10 μ moles, potassium phosphate buffer (pH 7.4), 150 μ moles, uracil 0.5 μ mole, cysteine, 10 μ moles, DPN, 0.05 mole, dihydrouracil dehydrogenase, 25 units, in a total volume of 3.0 ml. The control contained all components except dihydrouracil. Glucose dehydrogenase (250 units) and glucose (200 μ moles) were added as indicated.

position product showed the presence of a compound which behaved identically with β -ureidopropionic acid in the solvents employed.

To establish definitely that the enzymatic product was dihydrouracil,

its infrared absorption spectrum was determined and compared with that of authentic dihydrouracil. Fig. 2 shows that the enzymatic product has the same infrared absorption spectrum as dihydrouracil.

These data show clearly that the enzymatic product of uracil reduction is dihydrouracil.

Oxidation of Dihydrouracil to Uracil—The reversibility of the conversion of uracil to dihydrouracil was demonstrated by incubation of the purified enzyme with dihydrouracil under routine assay conditions, except that glucose and glucose dehydrogenase were omitted. A compound with the ultraviolet absorption spectrum of uracil was formed from dihydrouracil (Fig. 3), its complete removal was effected by the addition of glucose and glucose dehydrogenase (Fig. 4). The oxidation of DPNH was achieved by the action of a DPNH oxidase present in the enzyme preparation.

DISCUSSION

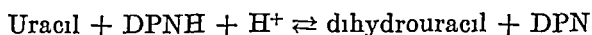
By the use of a purified enzyme preparation it was shown that the first step in the catabolism of uracil by *C. uracilicum* involves a DPNH-dependent reduction of uracil to dihydrouracil. That this is a reversible reaction was demonstrated by the quantitative oxidation of dihydrouracil to uracil with DPN. The purified enzyme is specific for uracil, not attacking cytosine, 5-methylcytosine, orotic acid, or thymine. It is also inactive in converting dihydrouracil, β -ureidopropionic acid, or carbamyl phosphate to end products.

The reduction of uracil to dihydrouracil is analogous to the reduction of orotic acid to dihydroorotic acid by enzyme preparations from *Zymobacterium oroticum* (7). Evidence for the reduction of thymine to dihydrothymine by rat liver slices has been presented by Fink *et al.* (13), although the requirement for DPNH was not tested and the reversibility of the reaction was not unequivocally demonstrated. The significance of the reductive pathway of pyrimidine metabolism was discussed earlier (2).

It appears that the dihydropyrimidines are important intermediates in the catabolism of pyrimidines by a variety of biological systems. What role they play, if any, in pyrimidine nucleotide synthesis is not yet known. Evidence, however, against their direct conversion to pyrimidine nucleotides has been presented by Lagerkvist *et al.* (14), who showed that dihydrouracil was inefficient as a direct precursor of mouse tumor nucleic acid. Green *et al.* (15) have also recently reported that, in *Escherichia coli*, dihydropyrimidines were inactive in transglycosidation reactions with thymidine under conditions whereby deoxyuridine was readily formed from uracil and thymidine.

SUMMARY

An enzyme, dihydrouacil dehydrogenase, isolated and partially purified from extracts of *Clostridium uracilicum*, was shown to catalyze the reaction



Certain properties of the enzyme were studied

The product of uracil reduction was isolated in crystalline form and identified as dihydrouacil by the following criteria paper chromatography, melting point determinations, elementary analysis, and ultraviolet and infrared absorption spectrophotometry

The author wishes to thank Dr. R. J. Foster of the Department of Agricultural Chemistry for running the infrared absorption spectra

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PYRIMIDINE METABOLISM

III THE INTERACTION OF THE CATABOLIC AND ANABOLIC PATHWAYS OF URACIL METABOLISM*

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(Received for publication, February 8, 1957)

A previous publication of this series had indicated the mechanisms, the requirements, and some of the properties of the enzyme systems in rat liver which degrade uracil and thymine (1). It was shown that during the enzymatic degradation of uracil the rate-limiting step is the initial reduction to 5,6-dihydrouracil¹. This property facilitates the measurement of the extent of the enzymatic degradation of uracil-2-C¹⁴, since the C¹⁴O₂ evolved becomes a direct measure of the amount of uracil degraded. It had subsequently been shown (2) that rat liver contains a uridine phosphorylase and a uridine kinase, indicating that there are pathways available to animal tissues for the anabolism of uracil to the nucleotide stage.

The experiments to be presented in this communication indicate that free uracil can be utilized for the synthesis of RNA by rat liver slices. Under certain conditions uracil is almost as effective a precursor for RNA synthesis as is uridine, uridine 5'-phosphate, or orotic acid. These results can be reconciled with apparently contradictory reports (3, 4) by taking into consideration the relative activities of the catabolic and anabolic mechanisms as well as the dilution effect on the added radioactive substrate by the endogenous pool.

Methods and Materials—In most of the experiments, male white rats weighing 180 to 230 gm were used. The experiments with mice and spontaneous mouse hepatomas were performed on C3H mice obtained through the courtesy of Dr. J. Walter Wilson of Brown University at Providence, to whom we are grateful. Liver slices were obtained by free-hand sectioning (5). The incubation medium consisted of a Krebs phosphate buffer (6), which had been modified by raising the phosphate buffer to 35 parts while the NaCl had been decreased to 67 parts. To avoid precipitation

* This investigation was supported in part by grants from the American Cancer Society and from the National Institutes of Health, Public Health Service. A preliminary report was presented in *Federation Proc.*, 14, 324 (1955).

¹ The abbreviations used are DHU, dihydrouracil, UMP, uridine 5'-monophosphate, RNA, ribonucleic acid, DNA, deoxyribonucleic acid, TPN and TPNH, triphosphopyridine nucleotide (oxidized and reduced forms, respectively).

of calcium phosphate, the CaCl_2 was decreased to 0.5 part. To 110.5 ml of this buffer were added 4 ml of 0.16 M pyruvate, 4 ml of 0.10 M L-glutamate, 7 ml of 0.10 M fumarate, and 5 ml of 0.10 M succinate, the solution was adjusted to pH 7.2 to 7.4 with NaOH. Increase in the concentration of phosphate and elimination of glucose from the medium were found necessary in order to avoid the drop in pH which occurred in the presence of the relatively large amounts of tissue used in these experiments.

Unless otherwise noted, 1.5 gm of liver slices were incubated with 5 ml of the buffer-substrate mixture described above for 90 minutes in a Dubnoff metabolic shaker. Experiments carried out in Warburg flasks with NaOH in the center well had demonstrated that the loss of radioactivity in the medium after incubation of liver slices with uracil-2- C^{14} could be accounted for as C^{14}O_2 trapped in NaOH during incubation and subsequently isolated as BaCO_3 (1). Accordingly in routine experiments, after incubation, an aliquot of the deproteinized supernatant fluid was plated and the conversion to C^{14}O_2 was determined by difference as compared to zero time controls.

At the end of the incubation period the samples were cooled in ice and concentrated perchloric acid was added to give a final concentration of 0.4 N. The samples were homogenized with an additional 5 ml of cold 0.4 N perchloric acid. Subsequent treatment was as described by Tyner *et al* (7).

The RNA nucleotides were prepared either after an initial salt extraction of the RNA and reprecipitation in ethanol, followed by alkali hydrolysis (7), or after overnight treatment of the protein with 0.1 N KOH. The mononucleotides were then isolated by column chromatography (8) and further purified by paper chromatography (9). Radioactivity measurements were made on the nucleotides which were eluted from the paper chromatograms. These eluates were plated on stainless steel planchets (Tracerlab, Inc.), dried in a desiccator under reduced pressure, and counted on a gas flow counter to ± 5 per cent accuracy. The spectra of the isolated uridylic and cytidylic acids compared well with those published in the literature (10).

The preparation of the uracil-2- C^{14} , uridine-2- C^{14} , and UMP-2- C^{14} used in these experiments has been described (2), each was of the same molar specific activity as the orotic acid-2- C^{14} from which each was prepared. When the substrate concentration was the experimental variable, a solution of the radioactive precursor was added to a solution of the same non-radioactive compound and the mixture was added to the incubation flask. The specific activity of the substrate was then calculated on the basis of the extent of the dilution by the non-radioactive carrier. The synthesis of dihydrouracil-2- C^{14} and of β -ureidopropionic acid labeled in the ureido group with C^{14} has been presented (1).

The intestinal mucosa was obtained by scraping the washed intestine of the rat. The equivalent of approximately 250 mg of a pooled suspen-

TABLE I
Incorporation of Uridine-2-C¹⁴, UMP-2-C¹⁴, and Orotic Acid-2-C¹⁴
into RNA of Rat Liver Slices*

Experi- ment No	2 C ¹⁴ precursor		Specific activity of substrate	Specific activity of uridylic acid in RNA	Per cent replac- ement of uridylic acid†	Specific activity of cyti- dylic acid in RNA	Per cent replac- ement of cytidylic acid	Added sub- strate degrad- ed to C ¹⁴ O ₂	Specific activity of uridylic acid Specific activity of cytidylic acid
		μmoles	c p m per μmole × 10 ⁻³	c p m per μmole		c p m per μmole		per cent	
1‡	Orotic acid	33	5 15	35	0 69	9 2	0 18	6	3 8
	UMP	33	5 15	42 5	0 82	7 5	0 14	11	5 7
	Uridine	33	5 15	42	0 815	9 4	0 18	9	4 5
2	Orotic acid	33	5 15	13 5	0 26	5 1	0 10	7	2 5
	UMP	33	5 15	10 6	0 23	1 4	0 02	11	7 5
	Uridine	33	5 15	14 8	0 29	3 2	0 07	12	4 6
3	Orotic acid	0 13	1200	930	0 077	36 0	0 003	30	25 5
	UMP	0 13	1200	74	0 006			95	
	Uridine	0 13	1200	44	0 0037			93	
4	Uridine	71	2 6	52	2 2	18 5	0 72	13	2 8
	"	35	5 15	74	1 45	19 5	0 38	26	3 8
	"	3 5	51 5	73	0 145	3 5	0 0068	95	20 8
	"	0 35	515 0	64	0 069	1 8	0 0004	93	35 7

* In these experiments 5 gm of rat liver slices were incubated in 10 ml of the modified Krebs phosphate buffer

† Per cent replacement = ((micromolar specific activity of the nucleotide isolated from RNA × 100)/(micromolar specific activity of the substrate added to the incubation mixture))

‡ Experiments 1 and 2 were performed at different times under the same conditions. These results are included to demonstrate the reproducibility within an experiment despite the variation in absolute values among experiments. The values for cytidylic acid have consistently shown wider discrepancies than those for uridylic acid.

sion of mucosa from three or four rats was added to each flask containing 4 ml of incubation medium.

Results

Table I, Experiment 4, shows the results obtained when increasing concentrations of uridine-2-C¹⁴ were incubated in the presence of rat liver

slices At low substrate concentrations the radioactivity added could be accounted for almost completely as $C^{14}O_2$. As would be expected, the proportion of the added substrate which was converted to $C^{14}O_2$ decreased with increasing substrate concentrations. Under the same conditions, the amount of uridine incorporated into RNA appeared to increase with increasing substrate concentrations, as can be seen from the per cent replacement column. This phenomenon may be a reflection of the endogenous pool size of inert uridine. The endogenous pool would alter the specific activity of the added radioactive precursor much more at low than at high precursor concentrations, this would naturally reflect on the replacement value. This apparent increase in the amount of uridine incorporated into RNA with increasing uridine concentrations may also be due to the continuous presence of substrate during the incubation period which occurs at high precursor concentrations, in contrast to the low and continuously diminishing substrate pool size at low precursor concentrations. Friedkin *et al* (11) have also described a similar increase of thymidine incorporation into DNA with increasing thymidine concentration. Whether these results represent a real stimulation of RNA synthesis in the presence of high substrate levels or whether they represent an experimental artifact is difficult to decide at present. The third fact which emerges from this experiment becomes apparent when the ratio of the molar specific activities of the uridylic and cytidylic acids in RNA is measured with increasing substrate concentration. This ratio varies from values as high as 30:1 to values as low as 3:1 at high uridine levels. Such variation is probably a reflection of the mass action law in the sense that more cytidylic acid is formed from uridine, as the uridine concentration is increased, and can in turn be incorporated into RNA. The latter results help to explain the variability of the uridylic to cytidylic ratios reported in the literature by various authors (12-14).

In Table I the relative rates of incorporation of orotic acid, UMP, and uridine into RNA also are included. The results are strictly comparable only within each experiment, as indicated by comparison of the results obtained in Experiments 1 and 2. In Table II are given the results obtained when uracil, UMP, and orotic acid, each labeled in carbon 2 with C^{14} , were incubated with rat liver slices during the same experiment. These results show the same general trend indicated by the results of Table I. It is interesting to note that, while the per cent replacement for the three substrates shows wide variations at low substrate levels, it becomes of the same order of magnitude when the substrate concentration is increased sufficiently. These results indicate the uncertainty involved in concluding that one or the other of these substrates is a better precursor for the uridylic acid of RNA.

In Tables III and IV are presented the experimental values obtained for the extent of incorporation of uracil-2-C¹⁴ into RNA, as well as the extent of degradation of uracil, dihydrouracil, and β -ureidopropionic acid by various tissues. Slices obtained from spontaneous mouse hepatomas, which incorporated uracil significantly into RNA, degraded uracil, dihydrouracil, and β -ureidopropionic acid to an appreciably lower extent than normal liver, only 10 per cent of the added uracil was degraded, as

TABLE II
*Incorporation of Uracil-2-C¹⁴, UMP-2-C¹⁴, and Orotic Acid-2-C¹⁴
into RNA of Rat Liver Slices**

2 C ¹⁴ substrate		Specific activity of substrate	Specific activity of uridylic acid in RNA	Replacement of uridylic acid†	Added substrate degraded to C ¹⁴ O ₂
	μM	$\text{c p m per } \mu\text{mole} \times 10^{-3}$	$\text{c p m per } \mu\text{mole}$	<i>per cent</i>	<i>per cent</i>
Orotic acid	30 0	10 0	62	0 62	9
	8 0	37 4	134	0 35	10
	2 0	150	450	0 30	12
	0 518	580	1380	0 24	20
UMP	30 0	10 3	69 5	0 68	15
	8 0	38 7	86	0 24	25
	2 0	93 0	46 5	0 05	92
	0 518	354	56	0 016	100
Uracil	30 0	18 7	66	0 35	22
	8 0	52 0	60	0 11	45
	2 0	140	47	0 034	90
	0 518	280	12 8	0 0045	98

* The experiment was performed as described in the text

† See Table I

compared to normal mouse liver slices, and also an appreciably decreased capacity to degrade DHU and β -ureidopropionic acid was demonstrated. Intestinal mucosa, which formed RNA extensively from these substrates, did not form C¹⁴O₂ at all from 2-labeled uracil and DHU, or β -ureidopropionic acid (ureido-C¹⁴).

Regenerating rat liver, as seen in Table III, exhibited only about one-fifth of the activity of normal liver in degrading uracil, but its ability to incorporate uracil into RNA, at specific times, was greater than that of normal liver. Thus, 48 hours after partial hepatectomy the ability of regenerating rat liver to incorporate uracil into RNA was about equal to that of the control, but 60 hours after the removal of the hepatic tissue

this value had increased to about 3 times that of the control This variation may be associated with the diurnal variation in the mitotic index of

TABLE III

*Incorporation of Uracil-2-C¹⁴ into Uridylic Acid in RNA by Various Tissues and Conversion of Uracil-2-C¹⁴ into Acid-Soluble Nucleotides**

Tissue	Conversion into acid soluble nucleotides†	Specific activity of uridylic acid in RNA
		c p m per μ mole
Rat liver	0 71	53
Regenerating rat liver 48 hrs after hepatectomy	0 65	58 5
“ “ “ 60 “ “ “	0 75	163
Rat intestinal mucosa	1 01	168
Mouse liver	0 68	76
“ hepatoma	0 75	540

* The experiment was performed as described in the text Uracil 2-C¹⁴ added was 0 518 μ M with a specific activity of 280,000 c p m per μ mole

† The results are expressed as per cent of the total uracil-2-C¹⁴ added to the incubation mixture

TABLE IV

Production of C¹⁴O₂ from 2-C¹⁴-Labeled Uracil and Dihydrouracil, and β -Ureidopropionic Acid (Ureido-C¹⁴) by Various Tissues†*

Additions		C ¹⁴ O ₂ production, per cent added radioactivity				
		Rat liver	Rat regenerating liver (48 and 60 hrs after hepatectomy)	Rat intestinal mucosa	Mouse liver	Mouse hepatoma
Uracil	γ 100	90	22	0	85	12
Dihydrouracil	300	95		0	74	21
β -Ureidopropionic acid	300	95		0	76	37

* The experiment was performed as described under “*Experimental*”

† Unpublished experiments by Skold, O, and Reichard, P (*personal communication*) indicate that, in addition to these tissues, Ehrlich ascites cells which incorporate uracil extensively also show a decreased capacity to degrade uracil

the liver which occurs in partially hepatectomized rats (15) Jardetzky *et al* (16) and Hecht and Potter (16) have shown that a diurnal variation in the incorporation of precursors into the DNA of the liver also occurs in partially hepatectomized mice This point will be dealt with more fully in the discussion

DISCUSSION

The high rate of replacement of RNA-uridylic acid by orotic acid as compared to the other substrates used at low substrate concentrations seems to be an artifact produced by at least two factors. One is the low orotic acid concentration in the tissue (17) and therefore the relatively small dilution by the meat orotic acid of the pool, the other may be related to the relatively lower capacity of the liver to degrade orotic acid. It is also possible that the UMP formed from orotic acid does not equilibrate with the tissue pool of UMP. This point bears further investigation. At high concentrations the dilution of the added substrates by the pool is small and the difference in incorporation rates becomes insignificant, at this point, even uracil, which has been considered not to be a precursor of RNA-uridylic acid, is incorporated to an extent which compares favorably with that of the other substrates tested. As has been discussed, the increased per cent replacement at high substrate concentrations may well be a reflection of the endogenous pool dilutions. That such a factor does in effect participate can be seen in Table II. The replacement figures for orotic acid increase only by a factor of 2.8 from very low to high substrate levels, while the corresponding increase in the replacement figures for UMP and for uracil are 42 and 77, respectively. In addition to this factor, an increase in the rate of synthesis of RNA also may be occurring at high substrate levels, as has been described by Friedkin *et al* for DNA (11). The present system cannot permit an accurate estimation of the relative significance of such a process. The extent of C^{14} incorporation into the cytidylic acid of RNA also appears to be dependent upon the substrate concentration, and this phenomenon may well be explained by a greater conversion of the substrate to free cytidylic acid at high substrate levels, this free cytidylic acid would then be subject to provisions similar to those described for the substrates tested. The extent of degradation to $C^{14}O_2$ provides, by difference, the amount of substrate remaining available for incorporation into RNA.

In the present investigation it has also been shown that tissues which incorporate uracil into RNA to an appreciable extent have a decreased capacity to degrade this compound, or the intermediates of uracil degradation, DHU, and β -ureidopropionic acid. Intestinal mucosa cannot degrade uracil even when the soluble extract fortified with TPNH is used. These conditions were used in order to obtain a fraction similar to that of rat liver and under conditions which have been demonstrated to be effective for the maximal activity of the degradative system (1). Of the tissues examined, intestinal mucosa and regenerating rat liver are rapidly growing tissues, since the cells of the intestinal mucosa are renewed about every 35 hours (18), while the regenerating rat liver doubles its weight in about

48 hours (19) The same cannot be said of the spontaneous mouse hepatoma This tissue, after its first appearance as a nodule, requires several months before it attains the weight of 1 gm² It would be erroneous, therefore, to group these tissues as rapidly growing tissues, although they do have in common a high rate of nucleic acid turnover and have been shown to incorporate free pyrimidines into their nucleic acids (4, 20-23)

The results obtained in these experiments indicate that there exists an inverse relationship between RNA turnover and the capacity of the tissue to degrade uracil In Paper II of this series (2), this balance of the degradative and anabolic pathways was referred to as a possible homeostatic mechanism From the present study it appears that this mechanism may aid in governing the rate of RNA synthesis, particularly since both the degradative and the anabolic pathways are present in the soluble cytoplasmic fraction of rat liver (2) This statement is further corroborated by the fact that the enzyme systems associated with RNA synthesis are also present in the same cellular fraction (24) From the results in Table II, and as previously discussed, it seems probable that an increase in the tissue concentration of the precursor can only increase the incorporation into RNA to a limited extent It is apparent, therefore, that some other factor must be involved in this process in which a decreased capacity for degradation is only the first step This statement is also supported by the results with regenerating liver shown in Table III The capacity of this tissue to incorporate uracil into RNA increases and decreases against a constant background of a diminished capacity to degrade uracil³ In other words, the decreased capacity to degrade uracil *per se* seems to be a necessary but not a sufficient cause for an increased incorporation of uracil into RNA

The cause for the variation of uracil incorporation into RNA seems to be associated with the diurnal variation of the mitotic index (15) This is also accompanied by a variation in the rate of DNA synthesis (16) The responsible agent for this variation may be related to a factor in the blood of partially hepatectomized rats (25) which causes an increase in the mitotic index of normal rat liver

SUMMARY

1 The incorporation of 2-C¹⁴-labeled uracil, uridine, uridine 5'-phosphate, and orotic acid into the ribonucleic acid (RNA) of various tissues has been studied

² Personal communication, Dr J W Wilson, Brown University, Providence, Rhode Island

³ The results obtained on the regenerating rat liver have been substantiated and extended with further experiments (to be published, E S Canellakis and J J Jaffe)

2 It has been shown that under certain conditions these compounds are incorporated into RNA at comparable rates

3 The ratio of the molar specific activities of uridylic acid to cytidylic acid in RNA is a variable dependent on the concentration of the substrate in the incubation medium

4 With increasing substrate concentrations there occurs an increased replacement of the uridylic acid of RNA. This phenomenon is discussed

5 Among various tissues examined, an inverse relationship between their capacity to degrade uracil and their capacity to incorporate uracil into RNA has been noted. It has been suggested that this may be part of a homeostatic mechanism which, in combination with other factors, regulates the rate of RNA synthesis

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COMPARISON OF THE AMINO ACID COMPOSITION OF T2 AND T3 BACTERIOPHAGES*

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The bacterial viruses of the T series (1) are composed of approximately equal amounts of nucleic acid and protein. This paper considers the question of whether two of these viruses, T2 and T3, which can attack the same host cell but which differ greatly in other biological properties, differ also in the amino acid composition of their proteins.

Methods and Materials

By using the apparatus (2) and medium (3) previously described, we have prepared two 4 to 5 liter batches of T2 bacteriophage by infection of the usual host, *Escherichia coli*, strain B. The raw titers were 1 and 3×10^{12} infectious T2 particles per ml. Purification of these lysates by three or four cycles of deliberately wasteful differential centrifugation yielded some 1.5 to 2 gm. each of lyophilized T2 bacteriophage which was dried in an Abderhalden drier at 56° (boiling acetone).

All batches were prepared from a master stock of T2 derived from a starting stock provided originally by Dr. Max Delbrück. The master stock and the purified T2 were typical in their behavior toward anti-T2 rabbit serum (1). As evidence of its biological homogeneity, the purified T2 was shown to give no extraneous plaque types and no more than the expected slight titer against *E. coli*, strain B/2, a host cell specifically resistant to T2. The purified T2 was also examined in the analytical ultracentrifuge and the electron microscope (4). Both tests indicated no discernible contaminants. In the electron microscope the phage appeared uniform and essentially free from empty membranes. The T3 preparation has been described previously (3).

The amino acid analyses were made chromatographically by the Moore and Stein technique (5, 6), with use of columns of Dowex 50, with slight modifications (3, 7). For each determination a 2 to 3 mg. sample of dried virus was accurately weighed into a 13 × 100 mm. acid-cleaned test tube and mixed with 0.5 ml. of glass-distilled, constant boiling hydrochloric

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acid The tube was frozen in liquid nitrogen, evacuated to 0.1 mm or less of mercury, and sealed The sealed section was drawn out and bent into a loop about 1 cm in diameter A group of such tubes was hung in a special hydrolysis apparatus, which consisted of a 2 liter Erlenmeyer flask provided with a 24/40 standard taper female joint and with internal "pegs" placed about 5 inches above the bottom and made by pushing locally heated glass inward (with a tungsten needle) about an inch and slightly upward, as in making a Vigreux distilling column The bottom of the flask was covered $\frac{1}{4}$ inch deep with 1,1,2-trichloroethane, b.p. 113.5, a condenser was inserted into the standard taper joint, and the tubes were maintained in the vapor of the refluxing solvent, for 16 to 110 hours After trying a number of commercial ovens and constant temperature baths, we have found this system to be the simplest for reproducible hydrolyses Each tube was removed and opened, the top portion being rinsed into the bottom with a few tenths ml of water The subsequent preparation of the samples for chromatography was the same as that described by Hirs, Stein, and Moore (7)

Results

The complete results of the present analyses are summarized in Table I, which compares the amino acid composition of T2 (eight determinations) with that of simultaneously hydrolyzed samples of T3 (five determinations) When the data are compared by use of the t distribution, it is concluded that the differences in the content of five of the amino acids are significant beyond a 99.1 probability: leucine ($t = 11.2$), histidine ($t = 8.6$), phenylalanine ($t = 5.9$), alanine ($t = 5.3$), and isoleucine ($t = 4.3$) Other differences of less significance can be shown, but the differences in these five amino acids alone are ample to support the essential point, *viz.*, that the same bacterial cell is capable of synthesizing viruses with appreciably different amino acid composition Although other interpretations are possible, it seems most likely that these differences are another expression of the genetic differences which are so manifest in their biological properties It is worth noting, however, that T2 and T3 are remarkably similar in the *general* pattern of amino acid composition to the host cell protein (8)

The previous paper on T3 amino acid composition (3) discussed in detail the reproducibility of the determinations under our conditions In the present work, in order to permit corrections to be applied for the decomposition of some of the amino acids during hydrolysis, samples were hydrolyzed for 16 and 72 or 110 hours It is well known that serine and threonine undergo appreciable degradation during hydrolysis (*cf.* Hirs, Stein, and Moore (7)) With the intact bacterial virus samples, however,

we find an even more pronounced decomposition of tyrosine. Samples of tobacco mosaic virus have not shown this effect under identical conditions, and it is reasonable to assume that it may arise from an interaction of the tyrosine with degradation products of the nucleic acid present in large amount in the bacterial virus. We have compensated for these losses by

TABLE I
*Amino Acid Composition of T2 and T3 Bacteriophages**

Amino acid	Amino acid per 100 gm. virus (dry weight)		
	T2 (8 determinations)	T3 (5 determinations)	Difference (T3 - T2)
	gm	gm	gm
Alanine	3.5 ± 0.14†	4.4 ± 0.09†	0.9†
Arginine	2.3 ± 0.21	2.8 ± 0.23	0.5
Aspartic acid	5.3 ± 0.11	5.4 ± 0.03	0.1
Cystine	<<1	<<1	
Glutamic acid	5.4 ± 0.11	5.3 ± 0.07	-0.1
Glycine	4.3†	3.8†	-0.5
Histidine	0.4 ± 0.02	0.8 ± 0.05	0.4†
Leucine	2.7 ± 0.10	4.4 ± 0.06	1.7†
Isoleucine	3.0 ± 0.04	2.2 ± 0.10	-0.8†
Lysine	2.9 ± 0.16	2.8 ± 0.20	-0.1
Methionine	1.0 ± 0.04	0.9 ± 0.03	-0.1
Phenylalanine	2.5 ± 0.11	1.6 ± 0.04	-0.9†
Proline	1.8 ± 0.09	2.1 ± 0.08	0.3
Serine	2.4†	1.9†	-0.5
Threonine	2.7†	3.2†	0.5
Tyrosine	2.9†	2.4†	-0.5
Valine	2.7 ± 0.12	3.0 ± 0.03	0.3
Total	45.7	47.0	

* Tryptophan and cysteine were not determined (see the text)

† Standard deviation of the mean. The values with no standard deviation were obtained by extrapolation (see the text)

‡ Significant difference with a probability of 99 to 1 or greater

extrapolation to zero hydrolysis time as described by Hirs, Stein, and Moore. We find the following per cent decomposition in 16 hours at 113° with constant boiling hydrochloric acid: serine, 9; threonine, 3.5; tyrosine, 27. Compensation for losses of other amino acids (7) has not seemed to be justified by our data.

An effect which is surely due to the use of intact virus rather than virus protein is the considerable increase in the yield of glycine with prolonged hydrolysis (9). Since it became obvious that under our hydrolysis condi-

OXIDATIVE PHOSPHORYLATION BY AN ENZYME COMPLEX FROM DISRUPTED BRAIN MITOCHONDRIA*

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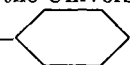
Although various attempts have been made to isolate a submitochondrial fraction capable of oxidative phosphorylation, it was not until the recent work of Cooper and Lehninger (1) that any degree of success was achieved. Among the techniques used for disrupting mitochondria have been ultrasonic disintegration, freezing-thawing, dispersion with a variety of chemical agents, and variations in pH values. Although slight oxidative phosphorylation (low P/O) was sometimes achieved (2), such treatment preserved only the oxidative portion of the system. Cooper and Lehninger (1) recently obtained a lipoprotein complex from digitonin extracts of rat liver mitochondria which was considerably more active than intact mitochondria on a weight basis.

The present study describes a multienzyme preparation from rat brain mitochondria which is similar to Cooper and Lehninger's Fraction P-1 (1) in many respects, but at the same time exhibits certain distinct differences. After work with a variety of dispersing agents, including digitonin, it was found that the detergent Triton¹ yielded the most active and consistent preparations. In addition to oxidizing such substrates as pyruvate and glutamate, the submitochondrial fraction from rat brain yields P/O ratios closely approaching those of intact mitochondria.

Methods

Preparation of Submitochondrial Phosphorylating Complex—Rat brain mitochondria were prepared after a modification of the method of Hogeboom *et al.* (3). Brains (usually four) from adult male Sprague-Dawley rats were washed in 0.25 M sucrose, homogenized thoroughly in 20 times their volume of 0.25 M sucrose in tight fitting glass homogenizers, and centrifuged twice at $1000 \times g$ for 5 minutes to remove nuclei, whole cells, and erythrocytes. The supernatant fluid was sedimented at $10,000 \times g$

* This research supported by a contract between the Office of Naval Research, Department of the Navy, and the University of Illinois.

¹ Triton WR-1339 Alkyl——O—(CH₂CH₂O)_n—CH₂CH₂OH, a non-ionic dispersing agent obtained from the Rohm and Haas Company, Philadelphia

in the Spinco model L ultracentrifuge for 15 minutes at 0° to yield a mitochondrial residue. After the mitochondria were washed in 20 volumes of 0.25 M sucrose and again sedimented at $12,000 \times g$ for 15 minutes, they were homogenized thoroughly in ice-cold 10 per cent Triton containing 10^{-4} M EDTA.² The mitochondria were then centrifuged at $80,000 \times g$ at 0° for 20 minutes. As in the case of Cooper and Lehninger's preparation (1), it was possible to obtain a clear supernatant fraction, a "loosely packed gelatinous" layer or "fluff," and a more tightly packed residue, R_1 . The fluff was suspended in 10 ml of 0.25 M sucrose containing 10^{-4} M EDTA and centrifuged at $100,000 \times g$ for 20 minutes to yield residue Fraction P-1 (after Lehninger's designation). When again centrifuged at $100,000 \times g$, the supernatant fluid yielded a residue P-2. After removal of P-2, the clear supernatant fluid was treated with 20 per cent its volume of 50 per cent trichloroacetic acid to yield R_2 for subsequent analysis. Both Fractions P-1 and P-2 were homogenized in 0.25 M sucrose containing 10^{-4} M EDTA, when used for enzymatic studies. Repeated extractions of R_1 yielded relatively inactive preparations of P-1 or P-2, and therefore only the first Triton extract was used in the present study.

Details of the technique used for measuring oxidative phosphorylation of brain mitochondria are described elsewhere (4). Phosphorylation was measured by determining the remaining orthophosphate at the completion of incubation by the method of Fiske and Subbarow (5). Acid-insoluble fractions were determined after the method of Schneider (6). The difference absorption spectrum of P-1 was determined exactly as described by Cooper and Lehninger (1), except that the system contained, in addition, 0.005 M magnesium chloride. The Beckman recording spectrophotometer DK 2 was used to obtain the absorption curve.

EXPERIMENTAL

Requirements for Oxidative Phosphorylation in Fraction P-1—In the presence of the complete system for measuring oxidative phosphorylation of intact brain mitochondria, it was possible to obtain P/O ratios of 2.3 (Table I). Although omission of magnesium did not completely abolish phosphorylation, both oxidation and phosphorylation were considerably depressed and the P/O ratio decreased 75 per cent. Neither DPN nor cytochrome *c* was necessary, whereas the omission of fluoride all but completely eliminated oxidation and phosphorylation. It was not possible to replace ADP with other nucleotides such as UDP, CDP, and GDP. UDP

² The following abbreviations were used: EDTA, ethylenediaminetetraacetate, ADP, CDP, UDP, and GDP, the diphosphates of adenosine, cytidine, uridine, and guanosine, respectively, DPNH, reduced diphosphopyridine nucleotide.

and CDP will partially replace exogenous ADP as phosphate acceptors when added to preparations of intact rat brain mitochondria³

TABLE I

Requirements for Oxidative Phosphorylation of Brain Mitochondrial Fraction

The values are an average of three experiments agreeing within 8 per cent. The complete system contained (final concentration) 0.008 M potassium phosphate, pH 7.5, 5×10^{-4} M K-ATP, 0.008 M $MgCl_2$, 0.008 M KF, 10^{-4} M DPN, 2×10^{-6} M cytochrome *c*, 0.02 M potassium pyruvate, 0.001 M potassium malate, 0.5 mg of yeast hexokinase, 0.004 M glucose, and P-1 (0.4 mg of N) in a total volume of 3.0 ml. Incubation was at 34° for 20 minutes; enzyme was tipped into the main compartment after 5 minutes incubation.

	ΔO microatoms	ΔP μ moles	P/O	Per cent inhibition of P/O
Complete	2.62	6.10	2.31	
No Mg	0.88	0.50	0.57	75
" DPN	2.40	5.50	2.28	2
" cytochrome <i>c</i>	2.25	5.10	2.27	3
" KF	0.80	0.19	0.23	90
CDP instead of ADP	0.20	0.00	0.0	100
GDP " " "	0.10	0.00	0.0	100
UDP " " "	0.10	0.00	0.0	100

TABLE II

Effect of Various Substrates on Oxidative Phosphorylation of Brain Mitochondrial Fraction

The final concentration of substrate = 0.02 M, except that DPNH = 0.005 M and cytochrome *c* = 10^{-4} M. The values are expressed as a mean of the number of experiments given, and the agreement within each set was usually 5 to 8 per cent.

Substrate	No. of experiments	ΔO	ΔP	P/O
		microatoms	μ moles	
Pyruvate + malate	8	2.62	6.10	2.31
Succinate	6	3.20	5.80	1.82
Glutamate	4	2.65	5.18	1.95
DPNH	3	1.85	2.50	1.35
Ascorbate + cytochrome <i>c</i>	3	5.00	2.85	0.57
None	2	0.00	0.00	0.00

Substrates Active with Fraction P-1—Many substrates which were utilized by intact brain mitochondria were active in the submitochondrial preparation (Table II). With succinate as a substrate, the P/O ratios were

³ Unpublished experiments, Abood, L. G.

almost 2.0, whereas with intact mitochondria the ratios were about 1.6, and oxygen consumption was generally lower than with pyruvate (4). Glutamate was readily oxidized, although the P/O ratio was less than that obtained with pyruvate. DPNH appeared to be more active in the P-1 preparation than with intact brain mitochondria (7). Cytochrome *c* plus ascorbic acid was also effectively utilized by the P-1 preparation, the P/O ratio obtained (0.57) is comparable to that observed with the complex from liver mitochondria (8). No endogenous oxidation or phosphorylation was observable.

TABLE III

Comparison of Effect of Various Inhibitors of Oxidative Phosphorylation on Intact Brain Mitochondria (M) and Submitochondrial Fraction P-1

The inhibitors were homogenized at 0° directly with the mitochondria or P-1 before addition to the flasks

Fraction		ΔO	ΔP	P/O	Per cent inhibition P/O
		<i>microatoms</i>	<i>μmoles</i>		
Control	P-1	2.58	6.02	2.32	
"	M	6.60	18.50	2.81	
CaCl ₂ , 0.002 M	P-1	1.20	2.50	2.08	10
	M	1.80	3.80	2.10	25
Phenyl ether, 5×10^{-5} M	P-1	2.52	5.65	2.25	4
	M	4.20	3.55	0.84	70
Reserpine, 5×10^{-5} M	P-1	1.00	0.46	0.46	80
	M	3.85	1.52	0.40	84
Dinitrophenol, 10^{-4} M	P-1	2.45	0.00	0.00	100
	M	6.40	0.00	0.00	100
Methylene blue, 5×10^{-5} M	P-1	1.82	0.00	0.00	100
	M	4.10	0.00	0.00	100

Comparison of Inhibitors on Intact and Disrupted Mitochondrial Preparations—Although calcium was somewhat less inhibitory to P-1 than to intact mitochondria, at a concentration of 0.002 M it was still a potent inhibitor in either system (Table III). Phenyl ether, however, which was an inhibitor of intact mitochondria (9), was completely ineffective on P-1 at concentrations producing 70 per cent inhibition of P/O in morphologically intact mitochondria. All other uncoupling agents studied, including 2,4-dinitrophenol and the tranquilizing drug, reserpine (9), were equally effective on both systems.

Difference Spectrum of Fraction P-1—The difference spectrum (10, 1) between the oxidized and reduced forms of P-1 reveals the presence of a number of components of the electron transport system (Fig. 1). Absorption maxima and minima were observed at wave lengths associated with

the α -, β -, and γ -peak of cytochrome *c*, the reduced forms of flavoprotein and cytochrome *a*, and α - and γ -peaks of the cyanide complex of cytochrome *a*,

Distribution of Acid-Insoluble Components in Various Fractions—The P-1 fraction was found to contain one-fourth of the total nitrogen present in the original mitochondrial preparation (Table IV). An appreciable amount (13 per cent) of the mitochondria ended up in Fraction R₃. One-fourth of the total nitrogen of the intact mitochondria was not accounted

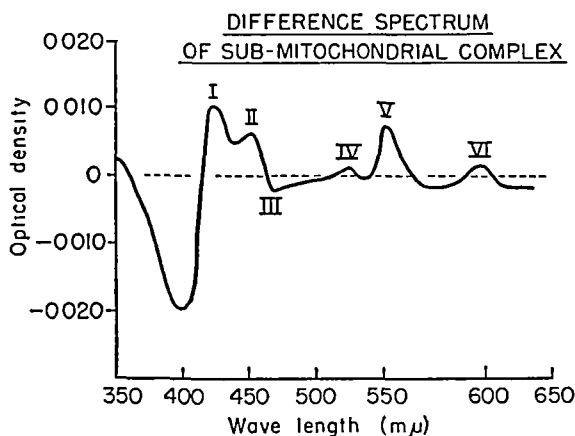


FIG 1 Difference spectrum of submitochondrial fraction, P-1. A cuvette containing 0.02 M potassium pyruvate, 0.001 M potassium malate, 0.005 M ADP, 0.01 M potassium phosphate (pH 0.5), 0.008 M $MgCl_2$, and 0.005 M KCN, and Fraction P-1 (0.5 mg of N) in a total volume of 2.8 ml, representing the reduced enzyme complex, was read against a "blank" cuvette containing everything except the substrates and cyanide. The reduced forms of the carriers are designated by roman numerals: I, γ -peak of cytochrome *c*, II, γ -peak of cytochrome *a* and cyanide complex of cytochrome *a*, III, reduced flavoprotein, IV, β -peak of reduced cytochromes *b* and *c*, V, α -peak of reduced cytochrome *c*, VI, α -peak of reduced cytochrome *a* and the cyanide complex of cytochrome *a*.

for in the combined residues. Of all the fractions, P-1 contained the highest content of phospholipide and nucleic acid. Approximately 85 per cent of the total phosphorus in P-1 could be accounted for on the basis of phospholipide, a figure which is comparable to that found for the liver complex (1). In so far as only about 75 per cent of the total mitochondrial nitrogen and nucleic acid could be accounted for, the remainder must be present as smaller molecular weight components in the soluble fraction.

Electron Micrographs⁴ of Mitochondrial Preparations—Morphologically intact brain mitochondria, prepared in isotonic sucrose, are spherical in shape and from 0.5 to 1.0 μ in diameter (Fig 2, a). The disintegrated

⁴ The authors are indebted to Dr J. P. Marbarger for the electron micrographs.

TABLE IV

Acid-Insoluble Components of Rat Brain Submitochondrial Fractions and Intact Mitochondria

The values are expressed in terms of mitochondria isolated from 1.0 gm. of wet weight of whole rat brain, and are an average of three determinations agreeing within 7 per cent.

Fraction	Dry weight		Nitrogen		Phospholipide		Nucleic acid (as nucleotide)
	Total	Per cent mitochondria	Total	Per cent mitochondria N	N	P	
	mg		μg		μg	μg	μg
Intact mitochondria	148		275		40	14.6	0.9
R ₁	78	53	94	34	25	3.4	0.28
P-1	25	17	67	24	7.0	4.6	0.26
P-2	3	2	73	2.7	1.1	0.7	0.05
R ₃	16	11	35	13	4.2	1.1	0.10

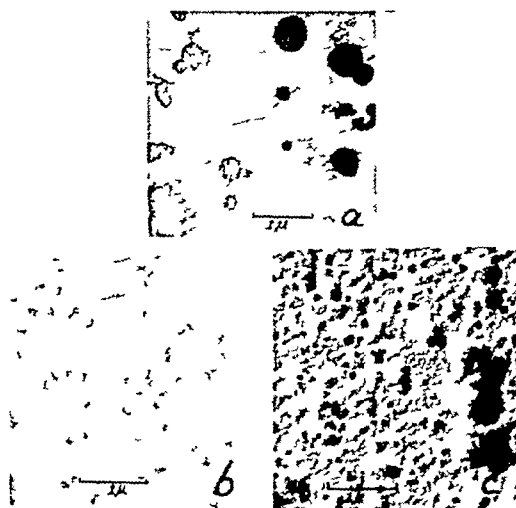


FIG 2a, 2b, and 2c. An electron micrograph⁴ (shadow cast) of intact brain mitochondria (2, a), of Fraction P-1 without shadow casting (2, b), and with shadow casting (2, c). The mitochondria and Fraction P-1 were suspended in 0.1 per cent osmic acid (adjusted to pH 7.0) for 15 minutes, and then washed consecutively with 50, 75, and 95 per cent ethanol. The final suspension was in 0.01 per cent Tween 80 (a detergent).

Fraction P-1 consists of heterogeneous particles varying in diameter from 0.1 to 0.01 μ (Fig 2, b). After shadow casting, the particles in Fraction P-1 appeared to be more or less spherical, although the presence of rather flattened large particles was occasionally apparent in some preparations.

(Fig 2, c) Intact mitochondria, or particles approaching mitochondria in size, were never observed in the microscope with any preparations of Fraction P-1

DISCUSSION

The present study is comparable to the work of Lehninger and co-workers (1) in that a submitochondrial fraction is found to be capable of carrying on oxidation and phosphorylation at a rate comparable to that of morphologically intact mitochondria. Although the particle size and weight, as adjudged by both electron microscopy and ultracentrifugal studies, appear to be the same for both preparations, most of the tricarboxylic acid components are still fairly well preserved in the brain preparation in contradistinction to Lehninger's liver fraction, where the only oxidases with appreciable activity were succinic acid and β -hydroxybutyric acid. At present, no explanation for this difference is apparent other than the possibility that treatment with digitonin was considerably more destructive than with Triton. Preliminary studies with digitonin extracts of brain mitochondria prepared according to Cooper and Lehninger (1) support this contention. Although such extracts behaved similarly to the Triton extracts, the activity was less than one-third. On the other hand, Triton extracts of liver mitochondria have yielded preparations which were similar to Lehninger's digitonin extracts³. It is not surprising that this difference exists between liver and brain mitochondria in view of their many chemical and biochemical distinctions (4, 7).

Another rather important difference between the brain and liver submitochondrial fractions concerns the effect of bivalent cations. Although the liver fraction requires no Mg^{++} , and is actually inhibited by Mg^{++} (1), the brain preparation will not function optimally without exogenous Mg^{++} . The elimination of EDTA from the preparation did not affect the requirement for Mg^{++} . While still acting as an effective uncoupling agent of the brain preparation, although somewhat less so than on intact brain mitochondria, Ca^{++} ions were not inhibitory to the enzyme complex of liver (1). In the case of brain mitochondria it is difficult to reconcile this uncoupling action of Ca^{++} to the concept that this cation acts by altering structural organization within mitochondria (11), since the brain preparations were already disrupted fragments. At this time, no explanation can be offered for the difference in either the Ca^{++} or Mg^{++} effect on the liver and brain submitochondrial preparations.

Other than the considerable difference in the particle size, the only outstanding difference thus far noted between the brain submitochondrial fraction and intact mitochondria is in the uncoupling effect of phenyl ether. Whereas phenyl ether is an extremely potent inhibitor of oxidative phos-

phorylation in intact brain mitochondria (9), it is completely inactive in the submitochondrial preparation at 100 times the concentration. This finding is analogous to the observation that thyroxine is not inhibitory to the liver enzyme complex (12). It would appear that the mechanism of action of phenyl ether on brain mitochondria is through an alteration of mitochondrial structure, as was demonstrated to be the case for the thyroxine inhibition of liver mitochondria (12). The similarity in the effect of phenyl ether and thyroxine is especially noteworthy in view of the fact that phenyl ether comprises the nucleus of the thyroxine molecule (9). An additional factor worth noting is that ADP appears to be the specific phosphate acceptor for the submitochondrial fraction from brain and liver (8), while UDP and CDP are substituted in part for exogenous ADP in intact mitochondrial preparations. It seems, therefore, that the action of CDP and UDP is mediated through ADP, and, once the endogenous ADP was removed from intact mitochondria through disruption, these nucleotides were no longer active.

The explanation for the metabolic differences of the enzyme complexes of liver and brain awaits further investigation. It is likely, however, that the phosphorylating complex of brain mitochondria is associated more intimately with the oxidases than is the case with liver mitochondria, when only two oxidases were present in the phosphorylating complex (1). With the possible exception of succinic oxidase it has not been possible to isolate a phosphorylating submitochondrial fraction from brain lacking in one or more of the oxidases present in intact mitochondria. With the diminution of phosphorylating activity during mitochondrial disruption, there appears to be a corresponding loss in the various oxidases.

SUMMARY

1 A submitochondrial fraction has been isolated from Triton extracts of brain mitochondria which is capable of carrying on oxidative phosphorylation with a variety of Krebs cycle intermediates.

2 The fraction is of a very small particle size, and contains many of the components of the electron transport system.

3 Mg^{++} and adenosine diphosphate (ADP) appear to be absolute requirements for oxidative phosphorylation, while exogenous cytochrome *c* and DPN are not. Other nucleotides will not replace ADP as phosphate acceptor.

4 The submitochondrial fraction from brain is compared with a preparation isolated from liver mitochondria by similar needs.

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ANALOGUES OF ACETYL CHYMOTRYPSIN

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Previous papers (1, 2) have reported the acylation of chymotrypsin with several nitrophenyl acetates. It was of obvious interest to test the behavior of nitrophenyl esters of other acids in the same connection. It now appears that chymotrypsin may be acylated by a number of acids differing considerably from acetic acid in structure. Benzoyl and salicyl chymotrypsins were prepared and found to resemble the acetyl derivative rather closely in so far as they were examined. On the other hand, hippuryl chymotrypsin forms and decomposes so rapidly that we have been unable to work with it. The stability, as well as the rate of formation of the acylated enzyme, has thus been found to vary with the nature of the acyl group, as might be expected if the acylated enzyme does indeed correspond to an intermediate in normal enzyme catalysis. A somewhat similar effect appears in the transesterification reaction of chymotrypsin (3), in that isobutyl and *tert*-butyl alcohols react very slowly in comparison to their straight chain analogues. Our attention was thus directed to the acids corresponding to those alcohols, namely isobutyric and trimethylacetic acids.

Materials and Methods

The nitrophenyl esters were made by the conventional methods which employ the appropriate acid chloride and pyridine. Hippuryl chloride was prepared according to Emil Fischer (4). After coupling, the reaction mixture was poured into dilute acetic acid and ice. The solid matter so obtained was dissolved in acetone and precipitated by the addition of water. If not crystalline, such precipitates became so upon standing at 5°. Three crystallizations were made in the same manner. The compounds were identified by melting point, when known, and by colorimetric estimation of the nitrophenol liberated on saponification with alkali.

The following analytical data were obtained with those compounds that have not to our knowledge been previously described.

2,4-Dinitrophenyl Benzoate—Found, C 54.3, H 2.80, 2,4-dinitrophenol 63.3, calculated, for $C_{13}H_8O_6N_2$, C 54.2, H 2.79, 2,4-dinitrophenol 63.8. Observed m p, 130–131°.

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p-Nitrophenyl Hippurate—Found, C 60.08, H 3.93, N (Dumas) 9.44, *p*-nitrophenol 46.3, calculated, for $C_{15}H_{12}O_6N_2$, C 60.0, H 4.00, N 9.34, *p*-nitrophenol 46.3 Observed m p, 170–171°

p-Nitrophenyl Isobutyrate—Found, C 57.4, H 5.37, N (Dumas) 6.75, *p*-nitrophenol 66.5, calculated, for $C_{10}H_{11}O_4N$, C 57.5, H 5.26, N 6.70, *p*-nitrophenol 64.4 Observed m p, 39–40°

p-Nitrophenyl Trimethylacetate—Found, C 59.3, H 5.84, N (Dumas) 6.30, *p*-nitrophenol 62.4, calculated, for $C_{11}H_{13}O_4N$, C 59.2, H 5.84, N 6.28, *p*-nitrophenol 62.5 Observed m p, 94–95°

p-Nitrophenyl Hydrocinnamate—Found, *p*-nitrophenol 52.2, calculated, for $C_{15}H_{13}O_4N$, *p*-nitrophenol 51.4 Observed m p, 97–98°

The acyl chymotrypsins¹ were prepared as previously described for acetyl chymotrypsin, with ether for the extraction of residual nitrophenyl ester and charcoal to remove nitrophenol (2). Neither isobutyryl, hydrocinnamoyl, nor hippuryl chymotrypsin was isolated from its solution, the first two were not required in these experiments, and hippuryl chymotrypsin was too unstable. *p*-Nitrophenylbenzene sulfonate reacted very slowly with chymotrypsin and was not studied further, *p*-nitrophenyl potassium sulfate² did not appear to react at all. Trimethylacetyl chymotrypsin crystallized readily from one-third saturated ammonium sulfate solution at pH 4.0, after an initial precipitation from the reaction mixture at two-thirds saturation. The crystals were cube-like but often occurred as twins. However, the substance decomposed during subsequent crystallizations, yielding typical crystals of the parent enzyme in active form. Spontaneous decomposition of trimethylacetyl chymotrypsin also took place upon storage, probably owing to traces of water absorbed by the protein after being dried in a vacuum (a common occurrence with proteins). After 30 days storage at 5°, the characteristic odor of trimethylacetic acid was pronounced, and the material had lost about half of its potential activity.

Results

Rate of Reaction Shown by Different Acyl Groups—The rate of reaction of several nitrophenyl esters with chymotrypsin is shown in Fig. 1.³ It is obvious that both the rate of acylation and the rate at which the acylated protein breaks down vary with the nature of the substituent group. In

¹ The molecular weight of chymotrypsin is taken as 25,000 in this paper.

² Prepared according to Burkhardt and Lapworth (5).

³ The reaction of 2,4-dinitrophenyl benzoate is not shown because this substance is quite insoluble and thus unsuited for colorimetric measurements. However, it was observed qualitatively to react with chymotrypsin at pH 5.0 when partly in suspension, so the benzoylated protein was prepared in the usual manner and has been used in some of the following experiments.

the short time occupied by these experiments, the breakdown of the trimethylacetyl complex was negligible

The slowness of the acylation by *p*-nitrophenyl trimethylacetate, to-

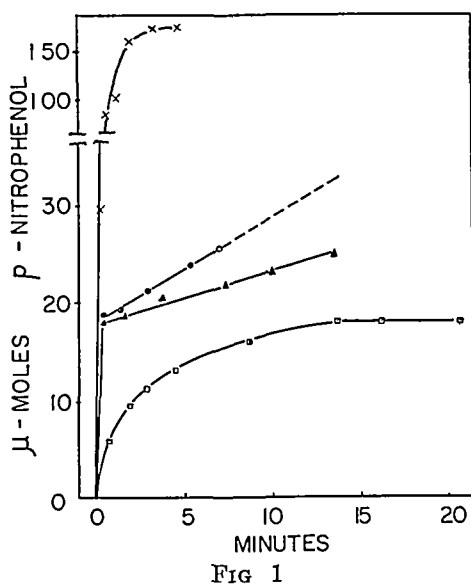


FIG 1

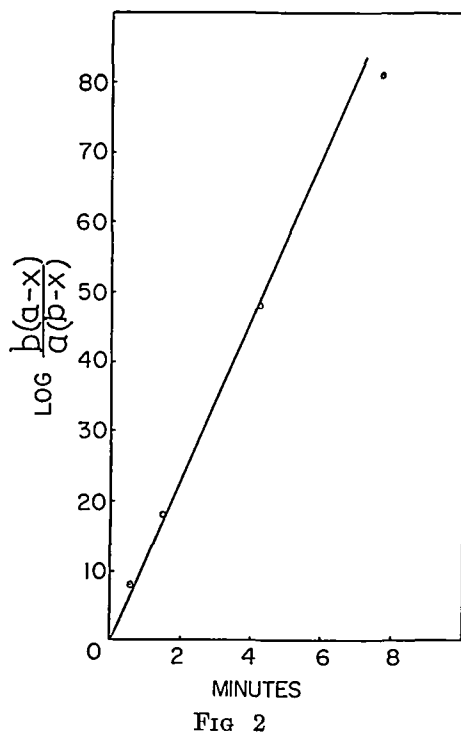


FIG 2

FIG 1 The rate of reaction (liberation of nitrophenol) between chymotrypsin and several nitrophenyl esters. Corrected for the spontaneous decomposition of the substrate, which was in all cases practically negligible. The system consisted of 10.0 ml of 0.07 M phosphate, pH 6.2, containing 4.5 mg (0.18 μ mole) of α -chymotrypsin and substrate as below. Temperature, 28°. \times = *p*-nitrophenyl hippurate (2.0 μ moles), \circ = *p*-nitrophenyl acetate (2.5 μ moles), \blacktriangle = *p*-nitrophenyl isobutyrate (2.5 μ moles), \square = *p*-nitrophenyl trimethylacetate (1.0 μ mole).

FIG 2 Showing agreement of the data for *p*-nitrophenyl trimethylacetate at pH 6.2 (from Fig 1) with second order reaction kinetics. a , initial molar concentration of substrate (1.0×10^{-4} M), b , initial concentration of free enzyme (1.8×10^{-5} M), x , concentration of acylated enzyme (taken as equal to concentration of free nitrophenol) at time t .

gether with the stability of the acylated enzyme, offers a good opportunity to study the kinetics of the reaction at relatively high pH levels. None of our experiments was designed with this in view or the ratios of enzyme to substrate would have been more appropriately chosen. Nevertheless, it may be of passing interest that the course of the reaction shown in Fig 1 for the trimethylacetate (at pH 6.2) follows second order kinetics until

about 80 per cent complete (Fig 2) Values for the initial velocities were also calculated from the apparent second order constants in other experiments at several pH levels The enzyme concentration in these cases was 6.5×10^{-6} M and the initial concentration of substrate was 10^{-4} M

TABLE I
*Hydrolysis of Acyl Chymotrypsin at 28°**

Ester used	Molarity	pH	Rate of hydro- lysis	Relative rate hydrolysis
	<i>mole per l</i>		<i>μmoles per min per mg enzyme</i>	
<i>p</i> -Nitrophenyl acetate	2.0×10^{-4}	6.20	0.0026	1.0
		6.70	0.0068	2.6
		7.20	0.013	5.0
		7.60	0.022	8.5
<i>p</i> -Nitrophenyl isobutyrate	2.0×10^{-4}	6.20	0.00096	0.37
		6.70	0.0029	1.1
		7.20	0.0040	1.5
		7.60	0.0064	2.5
<i>p</i> -Nitrophenyl trimethylacetate	1.0×10^{-4}	6.20	0.000084	0.032
		6.70	0.00018	0.069
		7.20	0.00029	0.11
		7.60	0.00040	0.15
<i>p</i> -Nitrophenyl hydrocinnamate	1.5×10^{-5}	6.20	0.041	16.0
<i>p</i> -Nitrophenyl hippurate	2.0×10^{-4}	6.20	0.27	100.0
L-Tyrosine ethyl ester	0.05	6.2	47†	18,000

* The initial reaction mixture consisted of 0.20 ml of substrate solution (in acetone), 0.90 to 4.5 mg of enzyme in 0.2 ml of water, and enough 0.07 M phosphate buffer of the desired pH to make a total volume of 10.0 ml The enzyme was added at zero time except in the case of the hippurate in which the substrate was introduced last

† The starting solution of 3.00 ml of 0.050 M L-tyrosine ethyl ester and 0.10 mg of α -chymotrypsin (introduced in 0.010 ml of water) was titrated at pH 6.2 with 0.355 N NaOH at 30° The rate is calculated to a temperature of 28° (assuming a Q_{10} of 2.0)

The values $\times 10^5$ were as follows, At pH 6.7, 4.9, at pH 7.2, 7.8, and at pH 7.6, 10 moles per second These values should give an approximate idea of the dependence of the speed of reaction upon pH Second order kinetics has been found to fit the analogous reaction with nitrophenyl acetate⁴ and the somewhat similar case of the inhibition of acetyl esterase by diisopropyl fluorophosphate (7)

⁴ In a private communication Dr Hans Neurath and Dr Gordon Dixon of the University of Washington have recently informed us of an elegant series of measure

The relative rates at which several acyl chymotrypsins were hydrolyzed at four pH levels appear in Table I. The rates were calculated from the later (steady state) portions of curves similar to those in Fig. 1 but usually

TABLE II
Hydroxamic Acid Formation

pH	Microequivalents of hydroxamic acid per μ mole (25 mg) chymotrypsin derivative		
	Acetyl	Benzoyl	Trimethylacetyl
5.0	0.4	0.6	
5.5	1.0		
6.0	1.1		
6.2		0.8	
6.7	0.9		
7.2			0
10.1			0
11.7	1.0	1.3	0.2

TABLE III
*Ratio of Milk-Clotting and Esterolytic Coefficients after Activation by Alkali**

	Time	TEE†	$K_m c$ ‡	$\frac{TEE}{K_m c}$
	days			
Chymotrypsin (3 times crystallized)		0.038	3.0	0.013
Benzoyl chymotrypsin	1	0.027	2.5	0.011
“ “	15	0.015	1.3	0.012
Acetyl “	217	0.038	3.0	0.013
“ “	120	0.031	2.6	0.012
Trimethylacetyl chymotrypsin§	10	0.030	2.7	0.011

* Tested 35 minutes after solution in 0.07 M phosphate buffer, pH 7.5

† Splitting of tyrosine ethyl ester under the conditions described previously (3) expressed as milliequivalents per minute per mg. of enzyme protein

‡ Milk-clotting activity by the method described previously (1) $K = 1/t \times \text{mg.}$, the reciprocal of the clotting time in minutes \times mg. of enzyme protein

§ pH 8.0, as described in Fig. 4. Results calculated to 25° on the assumption $Q_{10} = 2$

extended over a longer time. It may be noted that the rates are all pH-dependent.

The behavior of *p*-nitrophenyl hippurate, which appears to decompose so rapidly, is in fact rather sluggish when compared to that of a “good”

ments made at very short time intervals on the much less stable acetyl system. Reference is made to their thorough study which has now appeared (6).

ester substrate of chymotrypsin. Thus, at pH 6.2, L-tyrosine ethyl ester is hydrolyzed about 180 times as fast as *p*-nitrophenyl hippurate, and the latter, about 100 times as fast as the corresponding acetate. Although

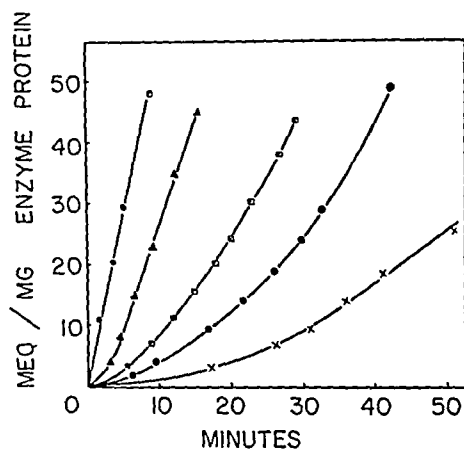


Fig 3

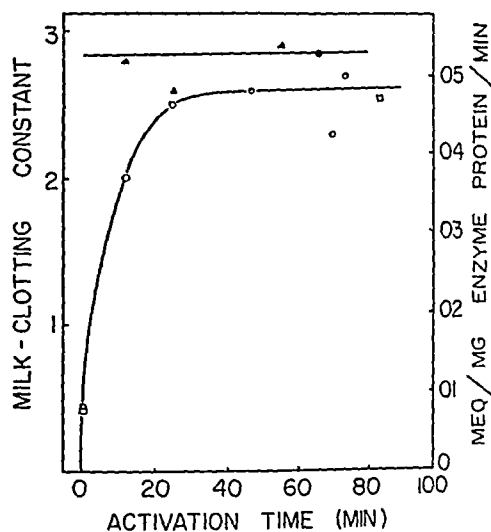


Fig 4

FIG 3 Reactivation in the presence of 0.05 M L-tyrosine ethyl ester, pH 6.2, at 30° and 0.025 mg of protein in a total volume of 30 ml. O = active chymotrypsin, \blacktriangle = acetyl chymotrypsin, \square = isobutyryl chymotrypsin, \bullet = benzoyl chymotrypsin (the results calculated from an experiment at 25° assuming $Q_{10} = 2$), and X = trimethylacetyl chymotrypsin.

FIG 4 Activation of trimethylacetyl chymotrypsin at pH 8.0. The initial reaction mixture was 9.7 mg of protein dissolved in 1 ml of 30 per cent glycerol and 70 per cent 0.1 M phosphate, pH 8.0, at about 25°. Suitable fractions of this solution were taken for esterolytic and milk-clotting tests, the latter containing an amount of active enzyme sufficient to clot 10 ml of pasteurized skim milk, pH about 6.5, containing 0.01 M CaCl_2 , in 1 to 2 minutes at 40° (1). The esterolytic activity is the usual zero order rate with tyrosine ethyl ester per mg of protein. The milk clotting constant is expressed as $1/t$ (minutes) per mg of protein. O = milk-clotting activity of the mixture containing trimethylacetyl chymotrypsin, \square = esterolytic activity of the same, \blacktriangle = milk-clotting activity, and \bullet = esterolytic activity of active (unacylated) chymotrypsin.

6.2 is optimal for the hydrolysis of the tyrosine ester, this is far from being the case with the other substrates.

Stability of Acylated Enzyme—Two criteria have been used: the pH level at which nearly complete reaction with hydroxylamine took place, (Table II) and the rate of the return of activity in mildly alkaline solutions (Table III). Hydroxamic acid formation, measured as previously described (2) (according to Hestrin (8)), is shown in Table II. There is no marked difference between the behavior of benzoyl and acetyl chymotryp-

sin On the other hand, trimethylacetyl chymotrypsin reacted incompletely, even at a pH level where such stable esters as ethyl acetate react quantitatively

It is evident from Fig 3 that, with the other acylated chymotrypsins tested, a substantial return of activity occurred in a few minutes in the presence of tyrosine ethyl ester at pH 6.2, but not with trimethylacetyl chymotrypsin. However, complete reactivation of this substance has been attained under the conditions described in Fig 4, in essence, when

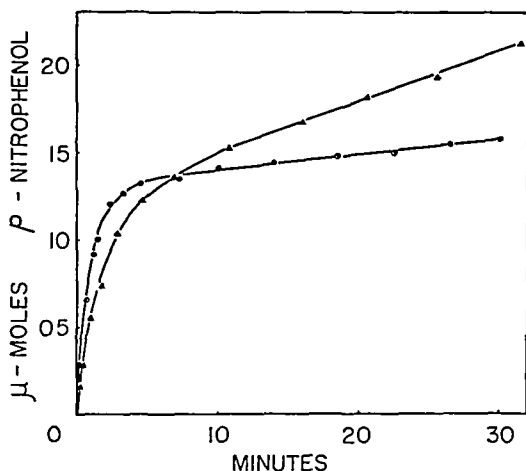


Fig 5 Transesterification reaction between *p*-nitrophenyl trimethylacetate and *n*-butanol catalyzed by chymotrypsin. Corrected for the (very small) spontaneous liberation of nitrophenol. The reaction mixture consisted of 10 ml of 0.07 M phosphate, pH 7.6, 1.0 μmole of *p*-nitrophenyl trimethylacetate (added in 0.2 ml of acetone), 4.0 mg (dry weight) of α -chymotrypsin, and *n*-butanol when used. Temperature, 23°. ○ = no butanol, and ▲ = 0.33 M *n*-butanol.

the inert protein was exposed to an unfavorably alkaline pH in the presence of glycerol to protect it from denaturation.⁵

It was of interest to see whether the reactivated protein possessed the same ratio of esterolytic and milk-clotting activities as the original. Table III shows that this was so in all the cases tested. Moreover, the spontaneous decomposition which sometimes occurs during storage affects both properties of the protein to the same extent.

Transesterification—No effort was made to study transesterification with all the chymotrypsin derivatives described here, but the outstanding stability of the trimethylacetyl derivative induced us to test this case. Fig 5 indicates that chymotrypsin mediates the transfer of trimethyl-

⁵ This scheme was not successful in reactivating a phosphorylated chymotrypsin (bis [*p*-chlorophenyl] phosphoryl chymotrypsin), although the exposure time was greatly lengthened.

acetyl to *n*-butanol, although when the pH and the quantity of enzyme used are considered, the rate is very much slower than in the case of acetyl

DISCUSSION

The existence of a wide variety of acylated chymotrypsins appears to be possible. Under otherwise comparable circumstances, both the rate of formation and the subsequent rate of decomposition (the stability) of the acylated protein obviously depend to a great extent upon the nature of the substituent group. The stability of these derivatives differs very greatly. One decomposes almost instantly (whereby the enzyme becomes a good catalyst), another exhibits stability approaching that of the phosphorylated chymotrypsins (whereby the "substrate" becomes a good inhibitor).

The marked lability of hippuryl chymotrypsin suggests that the presence of a peptide linkage in the acylating group decreases the stability of the complex. This appears to be a reasonable idea in view of the fact that the esters known to be most rapidly split by the enzyme do contain a peptide bond. The same conclusion may be reached if it is allowable to compare the behavior of the hippuryl with the benzoyl derivative, which is much less labile.

The marked stability of trimethylacetyl chymotrypsin suggests that the presence of a branched carbon chain in the acylating group tends to increase the stability of the acylated protein. The same conclusion is presented (though not so forcefully) by the behavior of isobutyryl chymotrypsin. There is also some evidence that stability in the phosphorylated chymotrypsins and phosphorylated choline esterases is likewise increased by the presence of a branched carbon chain (9). The opportunity is thus afforded through the nitrophenyl esters of preparing inhibitors of chymotrypsin and choline esterase of almost any desired potency. Some of these inhibitors offer obvious possibilities as insecticides and the like.

It is well known that both acids and alcohols of branched structure are esterified (by H^+ catalysis) with greater difficulty than their isomers with straight carbon chains. Moreover, the branched esters, once they are formed, undergo base hydrolysis less readily (10). Since the speed with which acyl chymotrypsins are reactivated increases greatly with OH^- concentration, the case is presumably one of base hydrolysis. Thus steric hindrance referable to the substrate itself may well be invoked to explain the stability of trimethylacetyl chymotrypsin. Just what role the configuration of the protein plays in this is not obvious, yet it must be an important one if the process is to be considered enzymatic. It may be noted that all of the nitrophenyl esters tested, with two exceptions (the sulfate and phosphate), reacted at least to some extent with chymotrypsin.

Clearly, there is no marked display of enzymatic specificity in these reactions

Yet there are some reasons for considering this process of acylation and deacylation as an example, perhaps a typical one, of enzymatic ester or peptide hydrolysis. Of the proteins known to catalyze the hydrolysis of nitrophenyl acetates, only those that are well recognized hydrolytic enzymes possess this property to any marked degree. In the most studied case, that of chymotrypsin, a single group essential to all the activity of the enzyme is preferentially acylated, and the enzyme is thereby inactivated so long as the combination lasts. The lack of specificity for the acylating group, which is so apparent with chymotrypsin, appears to be due to sole dependence of the reaction on the nitrophenyl group. However, it should be remembered that chymotrypsin is probably the most versatile hydrolytic enzyme to have been studied.

Another reason for considering the participation of the protein as important is the transesterification reaction (3) which may be observed best with chymotrypsin and was not observed to occur with such proteins as insulin and serum albumin. It is clear that in a transesterification chymotrypsin exhibits a decided preference for straight chain alcohols. Moreover, no such preference is shown by acetyl benzoyl histidine methyl ester (which is formed as an intermediate in the breakdown of nitrophenyl acetates by benzoyl histidine ester), although there is definite evidence of a slow transesterification reaction in the presence of free alcohols (11). The behavior of the protein, however, may be interpreted in opposite ways. Either the branched alcohol does not combine readily with the enzyme while the normal isomer does, or else both alcohols combine but the formation of the new ester is hindered in the case of the branched alcohol. The first case would indicate specificity on the part of the protein and would imply a special grouping as an anchorage for the alcohol. The second case might well be just another example of steric hindrance, with no implication of a particular binding place. We hope to decide the alternative soon.

SUMMARY

Chymotrypsin has been successfully acylated by a number of nitrophenyl esters whose acyl groups differ widely in structure. Both the speed of acylation and the stability of the resulting product varied with the nature of the acyl group. Both reactions were pH-dependent.

When the acylating group contained a peptide linkage (hippuryl chymotrypsin), the acylated protein was decidedly less stable than acetyl chymotrypsin. On the other hand, when the acyl group was an aliphatic acid with a branched carbon chain, a greatly increased stability in weakly

alkaline solution was observed. This was well illustrated by trimethylacetyl chymotrypsin. Compared with other acyl chymotrypsins tested, the substance formed slowly and decomposed very slowly, thus offering an excellent material for kinetic studies. It may be crystallized, it reacts incompletely with hydroxylamine even in strongly alkaline solutions, and it reverts slowly to chymotrypsin in the presence of tyrosine ethyl ester. It is readily reactivated at pH 8 under special conditions, when both the milk-clotting and the esterolytic properties of the original enzyme are recovered almost completely.

The existence of a great variety of acylated chymotrypsins, whose individual stability may vary between wide limits, appears possible. Thus, the very stable phosphorylated chymotrypsins are at one end of such a scale while substituent groups containing a peptide linkage appear to be near the other end.

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EFFECT OF VITAMIN B₁₂ DEFICIENCY AND FASTING ON THE INCORPORATION OF P³² INTO NUCLEIC ACIDS AND PHOSPHOLIPIDES OF INFANT RATS*

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Histochemical studies have shown a decreased concentration of cytoplasmic pentose nucleic acid¹ in the liver (1-4), pancreas (2), spinal cord (3), and cervical ganglia (3) of vitamin B₁₂-deficient rats. The concentration of both PNA and DNA per gm of tissue was decreased in livers from weanling rats fed a diet containing iodinated casein, but the amount per cell was unchanged (5, 6). Comparison of new born rats from control and vitamin B₁₂-depleted dams (7) showed no difference in the DNA or PNA concentration per gm of liver tissue, but the deficient livers contained slightly less PNA per cell. Brain tissue from the deficient offspring had more cells and more DNA per gm of tissue, but the amount of DNA per nucleus was unchanged. The amount of PNA per brain cell was less in the deficient animals. Recent observations on growing rats produced by vitamin B₁₂-depleted dams (8) confirmed earlier results that the concentration of DNA and PNA per gm of liver is decreased and demonstrated a decreased rate of PNA and DNA regeneration after partial hepatectomy.

From carcass analyses it has been postulated (9) that vitamin B₁₂ plays a role in carbohydrate and fat metabolism. An extension of this study (10) showed that a deficiency of vitamin B₁₂ results in a derangement of carbohydrate utilization and a decreased phospholipide content of blood and tissues.

In order to gain more insight into the effect of vitamin B₁₂ on nucleic acid and phospholipide metabolism, a study was made of P³² incorporation into brain and liver tissue of new born rats from vitamin B₁₂-depleted and control dams. The specific activity of the acid-soluble phosphorus de-

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¹ The following abbreviations are used: pentose nucleic acid, PNA; deoxypentose nucleic acid, DNA.

creased at a slower rate in the deficient animals. The vitamin B₁₂ deficiency had no effect on P³² incorporation into phospholipide, but markedly decreased its incorporation in both PNA and DNA. Fasting had a similar effect on nucleic acid synthesis, but also decreased P³² incorporation into phospholipide.

EXPERIMENTAL

The animals used were the new born offspring (usually less than 24 hours of age and weighing approximately 5 gm) of Wistar strain dams maintained for several months on a soy bean oil meal-glucose diet² (11) and controls that received a supplement of 30 γ of vitamin B₁₂ per kilo. The criteria used to determine a state of deficiency in the new born rat were described previously (12).

The isotope³ was administered by dorsal subcutaneous injection with a 1 inch, 27 gauge needle. The usual dose was 10 μ c of P³² given as the phosphate in a volume of about 0.1 ml. A few animals received 20 μ c. The activity of the original dose as well as that of the fractions was determined by use of a liquid dip counter, which was calibrated with a P³² standard obtained from the National Bureau of Standards. All counts were corrected for geometry and decay, and the maximal counting error was less than 5 per cent. Phosphorus was determined by an adaptation of the method of Fiske and Subbarow (13).

Unless otherwise noted, the animals were killed 12 hours after the isotope was administered. After decapitation, the tissues were removed, chilled on ice, blotted free from fluid, weighed (150 to 200 mg), and homogenized with cold water. The homogenate was precipitated with cold trichloroacetic acid and fractionated by the method of Schmidt and Thannhauser (14). After incubation in KOH, the chilled solution was acidified with HCl, treated with trichloroacetic acid, and the precipitate centrifuged. This residue was washed once with 2.5 per cent trichloroacetic acid and designated as the "DNA fraction." To the combined supernatant solution and washings were added 100 γ of phosphorus as KH₂PO₄, and the inorganic phosphorus was precipitated (15). The carrier phosphate was added to aid in quantitative removal of inorganic phosphorus from the supernatant solution, which was then designated as the "PNA fraction."

² Folic acid was supplied through the courtesy of Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, biotin through the courtesy of Dr. G. K. Parman, Hoffmann-La Roche, Inc., Nutley, New Jersey, and the other crystalline vitamins through the courtesy of Dr. L. Michaud, Merck and Company, Inc., Rahway, New Jersey.

³ The inorganic phosphate containing P³² was obtained from the Oak Ridge National Laboratory on allocation from the Atomic Energy Commission.

Of the total counts recovered, less than 5 per cent appeared in the phosphoprotein fraction, and this fraction will be neglected

The specific activity of all fractions was corrected for differences in dosage and body weight. As used here, specific activity is defined as counts per minute per microgram of phosphorus divided by the counts per minute administered per gm of body weight. In order to minimize the effect of different dosage levels and to relate the fractions more directly to their precursor, the activities of the lipid-soluble DNA and PNA fractions are reported as relative specific activities. The relative specific activity of a fraction is defined as the specific activity of that fraction divided by the specific activity of the acid-soluble fraction at the time of death.

Results

The distribution of P^{32} among the fractions of liver and brain tissue from deficient and control animals is shown in Table I. Although the PNA and DNA fractions prepared by the Schmidt-Thannhauser method are not of high radiopurity (16), the method allows recovery of essentially all of the phosphorus, and the results should be valid for the comparison under study. The animals used for these analyses were killed 12 hours after injection of the isotope. This period, although not optimal for nucleic acid incorporation studies in liver, was a compromise to allow incorporation into the brain tissue to approach the maximum, which requires about 48 hours in the mouse (17). The specific activity of the phosphorus in the acid-soluble fraction of both tissues from the deficient animals was higher than that in comparable tissue from controls. This is interpreted to mean that phosphorus is not utilized from the acid-soluble pool as rapidly in the deficient animals and that the phosphorus being incorporated into other compounds in the deficient animals at any given time is in equilibrium with a pool of higher specific activity than in the control animals.

The results do not allow one to determine whether the defect lies in the failure to utilize inorganic phosphorus or to utilize some other form of acid-soluble phosphorus. However, preliminary data (18) indicate that at the time of death about 65 to 75 per cent of the activity in the acid-soluble fraction of the livers and brains of deficient animals was due to inorganic phosphorus. In the control animals about 50 to 60 per cent was due to inorganic phosphorus. In animals killed at intervals from 1 to 12 hours after administration of P^{32} , the specific activity of the inorganic portion was approximately 50 per cent higher than that of the total acid-soluble fraction of liver. Over the period studied there was a constant relationship between these specific activities, regardless of the tissue or treatment. Daoust *et al* (19) also found that the specific activity of the inorganic phosphorus portion of the acid-soluble fraction of rat liver is

directly related to, and approximately the same as, the total fraction. It seems then that the specific activity of the phosphorus in the various fractions can logically be related to the acid-soluble fraction.

The relative specific activity of the lipid-soluble fractions in both liver and brain tissue was unchanged by the vitamin B₁₂ deficiency, but that of the nucleic acid fractions was decreased at least 20 per cent in both tissues. Thus, it appears that, while a vitamin B₁₂ deficiency in new born rats has

TABLE I

Vitamin B₁₂ Deficiency and Distribution of P³² in Brain and Liver Tissue

Twenty-six deficient and nineteen control animals about 6 hours of age received 10 μ c of P³² and were killed 12 hours later

Dietary supplement	Specific activity of acid soluble P* $\times 10^{-6}$	Relative specific activity†		
		Lipide soluble P	DNA P	PNA P
Liver				
None	768 \pm 6‡	0 930 \pm 0 026	0 344 \pm 0 013	0 186 \pm 0 013
Vitamin B ₁₂	598 \pm 23 <i>P</i> < 0 01§	0 934 \pm 0 019	0 437 \pm 0 016 <i>P</i> < 0 05	0 249 \pm 0 011 <i>P</i> < 0 01
Brain				
None	195 \pm 4	0 173 \pm 0 004	0 019 \pm 0 001	0 123 \pm 0 007
Vitamin B ₁₂	179 \pm 6 <i>P</i> < 0 02	0 185 \pm 0 005	0 028 \pm 0 002 <i>P</i> < 0 01	0 147 \pm 0 002 <i>P</i> < 0 01

* Specific activity = counts per minute per microgram of P divided by counts per minute administered per gm of body weight

† Relative specific activity equals specific activity of phosphorus in the fraction divided by the specific activity in the acid-soluble fraction

‡ Standard error of the mean

§ Statistical significance of the difference of the means

little or no direct effect on phospholipid synthesis, it does decrease the rate of synthesis of both DNA and PNA.

Since the vitamin B₁₂-deficient offspring commonly had less milk in their stomachs than the controls, it was important to determine whether the differences observed were simply the result of fasting. The effect of fasting on P³² incorporation in liver and brain is shown in Table II. In Trial I the animals were killed 12 hours, and in Trial II 24 hours, after administration of the isotope. In many respects the effect of fasting on P³² incorporation in liver tissue was analogous to that of the vitamin deficiency. The specific activity of the acid-soluble phosphorus was higher in the fasted animals than in the controls and the relative specific activities of the nucleic acid fractions were markedly lower. However, in contrast to the

vitamin B₁₂-deficient animals, 12 hours after treatment the relative specific activity of lipide-soluble phosphorus from the fasted animals was about one-third that of the controls. In the control animals killed after 24 hours, the relative specific activity of the lipide-soluble phosphorus in the controls was only slightly higher than those killed after 12 hours, whereas that in the fasted animals had doubled. This demonstrates that the rate of incorporation of phosphorus into phospholipide in the fasted animals was

TABLE II
Effect of Fasting on Distribution of P³² in Liver and Brain Tissue

Trial No	Conditions	Specific activity of acid soluble P* × 10 ⁻⁶	Relative specific activity		
			Lipide soluble P	DNA P	PNA P
Liver					
I†	Fasted (4)‡	913 ± 84	0.32 ± 0.02	0.15 ± 0.005	0.07 ± 0.003
	Control (3)	697 ± 42	1.00 ± 0.03	0.38 ± 0.011	0.30 ± 0.045
II§	Fasted (4)	678 ± 17	0.70 ± 0.03	0.33 ± 0.015	0.13 ± 0.009
	Control (4)	460 ± 8	1.10 ± 0.03	0.58 ± 0.030	0.43 ± 0.012
Brain					
I†	Fasted (4)‡	187 ± 14	0.056 ± 0.003	0.008 ± 0.002	0.046 ± 0.006
	Control (3)	178 ± 7	0.182 ± 0.011	0.016 ± 0.001	0.099 ± 0.010
II§	Fasted (4)	220 ± 6	0.136 ± 0.003	0.017 ± 0.001	0.091 ± 0.003
	Control (4)	245 ± 7	0.316 ± 0.009	0.042 ± 0.002	0.231 ± 0.007

* See Table I, footnotes

† 10 μ c of P³² injected at 6 hours into animals, fasted 18 hours, and killed 12 hours after injection

‡ Number of animals analyzed

§ 20 μ c of P³² injected at birth into animals, fasted 24 hours and killed

decreased and shows a distinct difference between the fasted and vitamin B₁₂-deficient animals

The effect of fasting on brain is analogous to that described for liver, except that fasting had no effect on the specific activity of the acid-soluble phosphorus. The tendency of fasting to decrease the relative specific activity of the lipide-soluble and DNA phosphorus was more marked in brain than in liver.

DISCUSSION

From the results of previous studies (1-8) it seems clear that a vitamin B₁₂ deficiency results in a decreased concentration of PNA per gm of tissue as well as per cell. The effect on the DNA concentration is not as clear, but it is doubtful that a deficiency affects the amount of DNA per nucleus

(7) Consequently, the concentration per gm of tissue will depend chiefly upon the size of the cells, since the deficiency has little effect on the moisture content of the tissues (12) The number of cells and the concentration of DNA in the brain tissue of new born rats deficient in vitamin B₁₂ are higher than normal (7) A similar effect on the number of cells per gm of brain has been obtained by fasting new born animals for 24 hours (18)

If the rate of incorporation of P³² into a tissue constituent may be used as a measure of the metabolic activity or turnover of that constituent, the turnover of the DNA and PNA P of both liver and brain tissue was depressed by a vitamin B₁₂ deficiency and by fasting It might be assumed that the effect of the vitamin deficiency was simply a fasting effect either from lack of food or failure to utilize the food, except for the fact that the deficiency had no effect on phospholipide metabolism, whereas fasting had a marked effect

The lower rate of DNA P turnover reflects a decreased rate of mitosis and it is difficult to distinguish cause and effect The lower rate of PNA P turnover is associated with slower rate of cell growth and, in general, with smaller cells This would account for the increased concentration of DNA per gm of brain tissue (7) The liver cells of new born rats undergo mitosis and growth in the same manner as regenerating liver cells It is interesting to note that 12 hours after injection of P³² into the normal new born animal the specific activity of the liver DNA P is higher than that of the PNA P The decreased rate of nucleic acid synthesis indicated by this study is in agreement with the observations of decreased regeneration of liver after partial hepatectomy in vitamin B₁₂-deficient rats (8) In relation to the rate of mitosis, it should be noted that the rate of P³² incorporation into brain DNA is a small fraction of that in liver, indicating a relatively slow production of new brain cells On the other hand, the incorporation of phosphorus into brain PNA proceeds at a more rapid rate, approximately one-half that in liver PNA This might be interpreted to mean that at this stage of growth the brain cells are increasing in size without a large increase in number It is also pointed out that at this age there is an appreciable incorporation of phosphorus into brain DNA, whereas in adult animals it is barely detectable (20)

The present results indicate that a vitamin B₁₂ deficiency did not affect the turnover rate of lipide phosphorus Ling and Chow (10) found less phospholipide in the tissues of vitamin B₁₂-deficient rats Although it is difficult to reconcile these observations, it should be pointed out that there was considerable age difference in the animals studied

SUMMARY

Radioactive phosphorus was administered to fasted new born rats and to the offspring of vitamin B₁₂-deficient dams The brains and livers were

fractionated and the specific activities of phosphorus in the various fractions were compared to similar fractions from control animals. The acid-soluble phosphorus had a higher specific activity in both the deficient and the fasted animals. The relative specific activities of the lipide-soluble phosphorus, deoxyribose nucleic acid (DNA) P, and ribose nucleic acid (RNA) P, related to acid-soluble fraction, were markedly lower in the fasted animals. The vitamin B₁₂-deficient tissues differed from those of the fasted animals chiefly in that there was no effect on the relative specific activity of the lipide-soluble phosphorus. In the vitamin B₁₂-deficient rat the metabolic activity of both DNA and RNA was lower than normal.

It was concluded that the lower relative specific activity of the RNA P in the vitamin B₁₂-deficient rat is related to a decreased rate of mitosis. The lower relative specific activity of the RNA P indicates a lower rate of RNA synthesis in the vitamin B₁₂-deficient animals and is associated with a small brain cell.

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THE METABOLISM OF D-GALACTOSE IN PSEUDOMONAS SACCHAROPHILA*

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New and radically different mechanisms for the oxidation of glucose and L- and D-arabinoses have been found recently in *Pseudomonas saccharophila* (1-5). In the present studies, a new pathway for the oxidation of D-galactose by the same organism is described. The proposed scheme is entirely different from the galactokinase-galactowaldenase pathway which has been worked out by Trucco *et al.* (6) and Caputto *et al.* (7-9), and combines some features of the metabolic mechanisms used by *P. saccharophila* for the oxidation of glucose on the one hand and of L-arabinose on the other. A preliminary report has been published (4).

Preliminary experiments showed that the "wild type strain" of *P. saccharophila* could be readily adapted to grow with galactose as substrate and that the adapted cells could oxidize galactose but could not use either glucose or fructose. The adaptation requires only a short period of time and is inductive in nature. Neither mutation nor the selection of adapted clones, which are prerequisites to the growth of the same species in glucose or fructose media (10), is necessary. In the presence of 0.002 M arsenite, adapted cells oxidize galactose with the production of pyruvic acid. When the cells are poisoned with 0.002 M iodoacetate, both pyruvic acid and methylglyoxal appear in the medium. These observations suggested that, as in the case of glucose metabolism, pyruvic acid and triose phosphate might arise from the splitting of a 2-keto-3-deoxy-6-phosphohexonic acid. In the following experiments, the evidence for such a reaction is presented and a sequence of biochemical steps leading to the formation of a new postulated phosphorylated intermediate is described.

Methods

Cultures of *P. saccharophila* were grown in a liquid mineral medium containing 2.5 gm of galactose, 1 gm of NH_4Cl , 0.5 gm of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg of ferric ammonium citrate, and 5 mg of CaCl_2 per liter of 0.033 M $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ buffer at pH 6.8. The cultures were incubated at 30° with continuous aeration on a rotary shaker. Cell-free extracts were

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† Fellow of the Rockefeller Foundation.

prepared by grinding the paste of centrifuged and washed cells with levigated alumina, extraction with 4 volumes of 0.01 M phosphate buffer, pH 6.8, and removal of the insoluble material by centrifugation (11). Crude preparations obtained in this manner could be stored for several weeks at -20° without appreciable loss of enzymatic activity.

D-Galactose was freed of glucose and purified according to the method of Palleroni *et al.* (12). Crystalline potassium D-galactonate was prepared from this purified galactose by hypiodite oxidation (13). 6-Phosphogalactonate was prepared by bromine oxidation from galactose 6-phosphate by R. Weimberg. Reduced diphosphopyridine nucleotide (DPNH) oxidase from *Azotobacter vinelandii* was partially purified by sedimenting a crude extract at $100,000 \times g$ for 2 hours¹. Calcium D-glyceraldehyde 3-phosphate and crystalline triosephosphate dehydrogenase were kindly provided by Dr. C. E. Ballou.

Lactones were determined according to Hestrin (14). With this procedure 1 μ mole of D-galactono- γ -lactone gives a reading of 120 with No. 54 filter in the Klett colorimeter, 1 μ mole of metasaccharin gives 100. Galactonic acid was determined by the same method after conversion of the acid to lactone by boiling for 5 minutes in N HCl. α -Keto acids were determined with the *o*-phenylenediamine reaction (15) or as semicarbazones (2). Pyruvic acid was determined according to the method of Friedemann and Haugen (16), toluene being used for the extraction of the hydrazone. Formaldehyde was determined by the method of MacFadyen (17). D-Glyceraldehyde was determined enzymically by measuring the reduction of DPN in the Beckman spectrophotometer at 340 m μ in a system containing 1 μ mole of DPN, 40 μ moles of sodium arsenate, 10 μ moles of neutralized cysteine, and 0.05 ml of a suspension of crystalline triosephosphate dehydrogenase in 3 ml of 0.026 M pyrophosphate buffer, pH 8.4. The Warburg respirometer was used for measuring oxygen consumption and CO₂ production. Radial, ascending, and descending chromatograms on acid-washed Whatman No. 1 filter paper were used for the identification and purification of various compounds. Before application on paper, the solutions were usually treated with either Amberlite IR-120 or Dowex 50 in the H form. The following solvents were used: Solvent 1, *n*-propanol-distilled formic acid-water (6:3:1), Solvent 2, methanol-distilled formic acid-water (80:13:7), Solvent 3, methanol-ammonia-water (6:1:3).

Alkaline silver nitrate spray (18) was used to detect substances capable of reducing silver. Brom. thymol blue and semicarbazide (19) sprays were used for the detection of acids and keto compounds, respectively. The *o*-phenylenediamine spray (15) proved to be the most specific for the detection of the α -keto acids (20).

¹ A. G. Marr, personal communication.

EXPERIMENTAL

In preliminary manometric experiments, in which crude cell-free extracts were incubated aerobically with galactose, DPN, and DPNH oxidase in tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 8.0, 0.5 mole of oxygen was taken up per mole of substrate. In the absence of either DPN or DPNH oxidase, galactose was not oxidized. All attempts to demonstrate a galactokinase with adenosine triphosphate (ATP) were unsuccessful. These observations suggested that the sugar is first oxidized to galactonic acid, possibly by way of a lactone. When galactonate and ATP were incubated with the enzyme, pyruvic acid was produced. If the re-

TABLE I
Formation of Pyruvate from Various Substrates

The reaction mixtures contained 0.2 ml of crude cell extract and 10 μ moles of $MgCl_2$ in a final volume of 1 ml of 0.05 M Tris-HCl buffer, pH 8.0, and were incubated for 1 hour at 30°. 10 μ moles of each substrate and of ATP and 6 μ moles of KF were added as indicated below.

Addition	Pyruvic acid formed
	μ moles
Galactonate	0
" + ATP	5.9
" + " + KF	6.9
6-Phosphogalactonate	2.9
" + KF	0
6-Phosphogluconate	9.0
" + KF	0
Gluconate	0
" + ATP	0

action was allowed to go to completion with an excess of ATP, 1 mole of pyruvate was formed per mole of galactonate initially added. The crude enzyme preparation also produced pyruvic acid from 6-phosphogalactonate and from 6-phosphogluconate. Neither of these compounds, however, fulfilled the requirements for being intermediates in the metabolism of galactonate. In the first place, 6-phosphogalactonate was decomposed at a much lower rate than was galactonate in the presence of ATP. Furthermore, the decomposition of both phosphohexonic acids was strongly inhibited by fluoride, while pyruvate formation from galactonate and ATP was practically unaffected (Table I).

It was further observed that, if galactonate was preincubated with the enzyme before the addition of ATP, the subsequent rate of pyruvate formation was doubled. During such preincubation galactonate was found

to disappear and a keto acid other than pyruvic acid to appear in the reaction mixtures

These findings led to the conclusion that an initial oxidation of galactose to galactonic acid is followed by a conversion of galactonic acid to a keto acid which can subsequently be phosphorylated and decomposed to yield pyruvic acid. Studies on some of the reactions in this sequence are detailed in the following sections

Oxidation of D-Galactose

With galactose as substrate, DPN was reduced by cell-free preparations in either phosphate, pH 7.0, or Tris buffer, pH 8.0. Triphosphopyridine nucleotide was reduced at approximately one-sixteenth the rate. L-Arabinose was also oxidized by preparations of cells grown with D-galactose, but the relative rates of DPN reduction with the two substrates varied greatly in different batches of cell-free extract. No oxidation of glucose, gluconate, or galactonate could be detected with either DPN or triphosphopyridine nucleotide as a hydrogen acceptor.

In a manometric experiment in which 0.2 ml of crude enzyme, 20 μ moles of D-galactose, 2 μ moles of DPN, and 0.1 ml of DPNH oxidase were incubated in a total volume of 2 ml of 0.05 M $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ buffer, pH 6.8, 10.2 μ moles of oxygen were taken up and no CO_2 was produced when the sugar had disappeared.

The initial product of oxidation appears to be D-galactono- γ -lactone. Under conditions similar to those described above, 11.9 μ moles of galactose were oxidized in 35 minutes at 30° and 4.76 μ moles of lactone were formed (calculated as galactono- γ -lactone). After 90 minutes of incubation, when 19.2 μ moles of galactose had disappeared, 5.16 μ moles of lactone were found. When neutralized hydroxylamine was used as a trapping agent for the lactone, a greater fraction of the oxidation product appeared as hydroxamic acid, even though the rate of oxidation was greatly reduced. In a mixture like the one described, but containing 100 μ moles of neutralized NH_2OH , 14.6 μ moles of galactose were oxidized in 170 minutes and 8.83 μ moles of hydroxamic acid were found.

The identity of the biologically produced lactone with D-galactono- γ -lactone was demonstrated by a comparison of the rates of both spontaneous and enzymatic hydrolysis of the two compounds, as will be shown in the following section.

Enzymatic Hydrolysis of Galactono- γ -lactone

When either chemically prepared galactono- γ -lactone or the product of the enzymatic oxidation of galactose was incubated with crude enzyme preparations in Tris or bicarbonate buffers at pH 6.8, they were found to

disappear at a rate much greater than that of the spontaneous hydrolysis of these compounds

When 80 μ moles of authentic γ -lactone were incubated at 25° with 0.8 ml of crude enzyme in 4 ml of 0.1 M phosphate buffer at pH 6.8, about 60 per cent of the lactone disappeared in 2 hours. Under similar conditions, but in the absence of enzyme or with boiled enzyme, less than 10 per cent was decomposed. That the product of enzymatic decomposition of the γ -lactone is galactonic acid was evident from the observations that poorly buffered reaction mixtures rapidly became acidic and that a large fraction of the product could be lactonized again to galactono- γ -lactone by heating with N HCl. In addition, the product of the reaction behaved identically with D-galactonic acid on chromatograms (Solvents 1, 2, and 3).

The following experiment was devised to demonstrate the identity of galactono- γ -lactone with the product of the enzymatic oxidation of galactose on the one hand and with the substrate for the delactonizing enzyme on the other. A reaction mixture containing 240 μ moles of galactose, 1.2 ml of crude enzyme, 12 μ moles of DPN, and 0.6 ml of DPNH oxidase in a total volume of 12 ml of 0.05 M phosphate buffer, pH 6.8, was incubated aerobically on a shaker for 90 minutes at 30°. The mixture was chilled and treated with Amberlite IR-120 in the H form. The proteins were quickly removed by centrifugation, the supernatant fluid was cautiously adjusted to pH 6.8 with NaOH and again clarified by centrifugation. Analysis showed that 223 μ moles of galactose had disappeared and 58 μ moles of lactone were present. A control mixture was then prepared, containing enzyme, DPN, DPNH oxidase, and the expected products of the reaction (17 μ moles of galactose, 166 μ moles of potassium galactonate, and 58 μ moles of authentic galactono- γ -lactone). This mixture was immediately deproteinized and neutralized. The rates of spontaneous hydrolysis at pH 6.8 and 8 and of the enzymatic hydrolysis at pH 6.8 of the lactones were then compared in the two samples (Table II).

Conversion of Galactonic Acid

With crude preparations, the rate of the phosphorylative reaction in which pyruvate is produced from galactonate could be doubled by preincubating the enzyme with galactonate before the addition of ATP. In the presence of excess ATP, 1 mole of pyruvate was formed per mole of galactonate initially added. During the preincubation period, the disappearance of galactonate could be demonstrated by the progressive decrease in the intensity of the hydroxamic acid reaction when samples of the solution were heated with N HCl and treated with hydroxylamine. At the same time, the formation of an α -keto acid was evidenced by the appearance of a substance which reacted with *o*-phenylenediamine and could be

decarboxylated with ceric sulfate. This compound did not form an extractable hydrazone when treated by the method of Friedemann and Haugen (16). On chromatograms with Solvent 1, the new compound has an R_F value of 0.57. (Galactonic acid had an R_F of 0.45 under the same conditions.) When sprayed with *o*-phenylenediamine-HCl and dried, it gives a yellow color with a greenish fluorescence in ultraviolet light. After heating at approximately 95°, the color changes first to a yellow-green and then to red. After standing for several hours at room temperature, the color becomes violet. In these color reactions, the compound resembles 2-keto

TABLE II

Spontaneous and Enzymatic Hydrolyses of Authentic and Enzymatically Prepared Galactonolactones

The reaction mixtures contained 20 μ moles of either lactone in a total volume of 5 ml. of 0.05 M phosphate buffer at pH 6.8 or 8.0, respectively. For enzymatic hydrolysis, 0.25 ml. of crude enzyme was included in the mixtures at pH 6.8 which were incubated at 30°. The average values of the hydrolysis constant (k) under the specified conditions were computed from determinations on several samples of each mixture taken after various periods of incubation.

$$k = \left(\frac{2.3}{t(\text{in hrs})} \right) \left(\log \frac{\text{initial concentration of lactone}}{\text{concentration of remaining lactone}} \right)$$

	k	
	Authentic galactono- γ lactone	Enzymatically prepared lactone
Spontaneous hydrolysis, pH 8.0	0.29	0.27
“ “ “ 6.8	0.06	0.06
Enzymatic hydrolysis, pH 6.8	0.28	0.24

3-deoxy-6-phosphogluconate (20). The amounts of keto acid produced were estimated with the *o*-phenylenediamine method (15), which was originally standardized against values obtained from the decarboxylation with ceric sulfate. Later the method was standardized with the crystalline potassium salt of the keto acid. The extinction coefficient of the quinova-line derivative was found to be $5.58 \times 10^6 \text{ cm}^2$ per mole at 330 $m\mu$. At least 90 per cent of the galactonate could be converted to keto acid after prolonged incubation. The optimum pH for the reaction was found to be about 8.0, the rate falling off to about two thirds of the maximal at pH 7.0 and 9.0. With crude extracts, the addition of magnesium did not increase the rate of reaction. No requirement for phosphate could be demonstrated, since enzyme preparations dialyzed against bicarbonate were still active when tested in Tris or bicarbonate buffer.

Preparation and Characterization of Keto Acid

1 gm of potassium galactonate was dissolved in 40 ml of solution containing 12.6 ml of 0.1 M phosphate buffer, pH 7.6, and 8 ml of crude enzyme. The mixture was incubated at 30° for 12 hours, during which time approximately 91 per cent of the substrate was converted. The solution was acidified and deproteinized by passage through a column of Dowex 50 in the H form and adjusted to pH 7.2 with a slurry of $\text{Ca}(\text{OH})_2$. Calcium phosphate was removed by centrifugation, the supernatant fluid was concentrated *in vacuo* to 4 ml, and 20 ml of methanol were added. A precipitate (200 mg), which formed overnight at 5°, was removed by centrifugation. (This precipitate, which contained 163 mg of the keto acid, much of the unchanged galactonate, and other impurities, was later purified by chromatography and crystallization of the potassium salt of the keto acid.) An equal volume of dry ether was added to the supernatant liquid and the mixture was left overnight at 5°. The precipitate which formed was centrifuged, washed with dry ether, and dried *in vacuo*. The yield was 550 mg of the calcium salt (95 to 97 per cent purity as $\text{Ca}(\text{C}_6\text{H}_9\text{O}_6)_2$). The calcium salt was redissolved, the solution was passed through a column of Dowex 50 in the H form, neutralized with KOH, and concentrated to 2 ml *in vacuo*. A mixture of absolute ethanol and ether (1:1) was added until a faint turbidity appeared. Crystallization of the potassium salt occurred overnight at 5°. The crystals were collected by filtration, washed with ether, and dried *in vacuo*. The yield was 420 mg. The crystalline potassium salt still contained a trace of galactonate, which could be detected by paper chromatography. For some of the more critical analyses to be reported, the keto acid was further purified by chromatography with Solvent 1 and recrystallized several times as the potassium salt from solutions in 80 per cent methanol by the addition of ether. Attempts to crystallize the free acid or the calcium salt were unsuccessful. The sodium salt forms very small crystals. The potassium salt decomposes with charring at 159–163°. It does not lose weight when kept under a vacuum over P_2O_5 for several days at room temperature or for 2 hours at 110°. Elementary analysis was as follows: C, 33.27 per cent, H, 4.28 per cent (theoretical for $\text{KC}_6\text{H}_9\text{O}_6$, C, 33.3 per cent, H, 4.17 per cent).

Ceric sulfate decarboxylation yielded 1.04 μmoles of CO_2 per μmole of the compound, as determined manometrically (21). No CO_2 was liberated with 4-aminoantipyrine, which decomposes β -keto acids (22). The ultraviolet absorption spectrum of the salt is very similar to that of pyruvate, with a strong absorption below 290 $\text{m}\mu$ and a slight shoulder with a maximum at 330 $\text{m}\mu$ (molecular extinction, $9.27 \times 10^3 \text{ cm}^2$ per mole at 330 $\text{m}\mu$). The compound gives a faint purplish color after 24 hours when tested with the cysteine-carbazole reaction (23) and no color with diphenylamine (24).

or cysteine and sulfuric acid (25) It gives a very faint pink color with the resorcinol reagent of Roe (26) and with the tryptophan reagent of Cohen (27) after prolonged boiling It reduces alkaline ferricyanide, according to the procedure of Schales and Schales (28) 1 μ mole has a reducing power equivalent to 0.78 μ mole of glucose

The compound forms a derivative with *o*-phenylenediamine with an absorption maximum at 330 $m\mu$ typical of quinoxaline (molecular extinction coefficient, 5.58×10^6 cm^2 per mole at 330 $m\mu$) The semicarbazone (2) also has an absorption spectrum characteristic of α -keto acid derivatives with a maximum at 250 $m\mu$ (molecular extinction coefficient, 1.01×10^7 cm^2 per mole at 250 $m\mu$) The highly purified compound can be lactonized to some extent by boiling for 5 minutes with *N* HCl After this treatment, however, only a faint color is obtained in the reaction with hydroxylamine and ferric salts (14) (1.0 μ mole gives a hydroxamic acid reaction equivalent to 0.09 μ mole of galactono- γ -lactone)

When 6 μ moles of the compound were incubated with either 25 or 40 μ moles of periodate at pH 1.6 in a total volume of 5 ml at 30°, 2.0 μ moles of periodate were reduced per μ mole of substrate after 1 hour More periodate was subsequently reduced at a low rate (0.11 μ mole per hour) At higher pH values, much greater rates of periodate reduction were observed and more periodate was consumed In all cases, 1.0 μ mole of formaldehyde was produced per μ mole of substrate initially added

All of the above observations support the conclusion that the compound is a 2-keto-3-deoxyhexonic acid formed by the dehydration of galactonic acid in much the same manner that 2-keto-3-deoxy-6-phosphogluconic acid is produced from 6-phosphogluconic acid (2) The α -keto configuration is supported by the reactions with ceric sulfate, *o*-phenylenediamine, and semicarbazide The occurrence of hydroxyl groups on the 3 terminal carbon atoms is shown by the reduction of 2 moles of periodate with the formation of formaldehyde The stereochemical configuration about carbon atoms 4 and 5 would be expected to be the same as that in galactose from which the compound is derived This expectation was justified by the following experiments, in which the biologically active compound has been synthesized from metasaccharin

Chemical Synthesis of 2-Keto-3-deoxygalactonic and 2-Keto-3-deoxygluconic Acids

α -Hydroxy acids are known to be oxidized to the corresponding α -keto acids in acid medium with chlorate and vanadium pentoxide Thus, gluconate, galactonate, and 6-phosphogluconate are converted to 2-ketogluconate, 2-ketogalactonate, and 2-keto-6-phosphogluconate, respectively (29, 30) This method was used to oxidize metasaccharinic (3-

deoxygalactonic) and D-glucometasaccharinic (3-deoxygluconic) acids to the corresponding α -keto derivatives. Crystalline metasaccharin (3-deoxy-D-galactonolactone) was prepared by a slight modification of the method of Evans *et al* (31). 1 mmole of metasaccharin was dissolved in hot water, neutralized with 5 N KOH while hot, and made up to 1 ml volume. 41 mg of KClO_3 , 2.5 mg of V_2O_5 , and 0.015 ml of phosphoric acid were added and the mixture was left at room temperature for 36 hours. The volume was made up to 10 ml with water and chlorate was eliminated by passing a stream of SO_2 through the mixture. The solution was next treated with Amberlite IR-120 in the H form, filtered, and aerated to remove excess SO_2 . The pH was carefully adjusted to 7.5 with a slurry of $\text{Ca}(\text{OH})_2$ and the solution was left overnight at 5°. Insoluble material was removed by centrifugation and the supernatant fluid was concentrated to 5 ml *in vacuo*. The calcium salt was precipitated and converted to the potassium salt by the procedure which has been described for the crystallization of the 2-keto-3-deoxyhexonic acid obtained enzymatically. The precipitate contained 263 μmoles of the salt of the keto acid (computed as the potassium salt of 2-keto-3-deoxyhexonic acid) and 87 μmoles of potassium metasaccharinate. The synthetic keto acid was indistinguishable from the enzymatic product on chromatograms developed with Solvents 1, 2, and 3 and sprayed with the *o*-phenylenediamine reagent. Attempts to separate it from metasaccharinic acid by chromatography were not successful.

The glucose analogue of the keto acid was next prepared from glucometasaccharin. A syrup of D-glucometasaccharin was prepared by the method of Sowden (32). This was treated with chlorate, V_2O_5 , and H_3PO_4 in the same manner as metasaccharin and the potassium salt of the keto acid was precipitated as before. When chromatographed with Solvent 1, the major product had the same R_F value and gave the same color reactions and fluorescence with the *o*-phenylenediamine reagent as the keto acid obtained from metasaccharin. Another unidentified keto acid, with an R_F of 0.76, was also present. The mixture was chromatographed and the glucose analogue of 2-keto-3-deoxygalactonic acid was eluted and converted to the potassium salt. The product was contaminated with glucometasaccharinic acid, but no effort was made to separate the two. The impure synthetic compounds were next subjected to enzymatic decomposition in the presence of ATP. The product obtained from metasaccharin was cleaved, yielding 0.97 μmole of pyruvate per μmole of keto acid. The analogous product obtained from glucometasaccharin was inactive, but not inhibitory to the enzyme. Neither of the parent saccharinic acids was attacked by the enzyme. These observations support the contention that the keto acid produced enzymatically from galactonic acid is 2-keto-3-deoxygalactonic acid.

Decomposition of 2-Keto-3-deoxygalactonate

1 μ mole of pyruvate is produced per μ mole of the crystalline keto acid when the latter is incubated with enzyme and an excess of ATP. Pyruvic acid was identified by its behavior in the Friedemann and Haugen reaction (specific toluene extraction) by paper chromatography (Solvent 1) and by the oxidation of DPNH in the presence of lactic dehydrogenase. Glucuronate was not converted to keto acids in the presence or absence of ATP by enzyme preparations of galactose-grown cells.

In preliminary experiments, evidence was obtained that the other product of cleavage is D-glyceraldehyde 3-phosphate, since DPN was reduced by the enzyme preparations in the presence of arsenate and cysteine, but not in the absence of these addition substances. However, as had been shown previously, triose phosphate is rapidly decomposed by crude preparations (1). In the following experiment triose phosphate was trapped with hydrazine and estimated with triosephosphate dehydrogenase. 3 μ moles of potassium 2-keto-3-deoxygalactonate were incubated with 10 μ moles of MgSO_4 , 10 μ moles of ATP, 15 μ moles of neutralized hydrazine, and 0.3 ml of crude enzyme in a total volume of 3 ml of 0.02 M Tris buffer, pH 8.0, for 20 minutes at 30°. The reaction mixture was deproteinized by treatment with Amberlite IR-120 in the H form and centrifugation. The hydrazone was decomposed by shaking with four successive 1 ml portions of benzaldehyde. Excess benzaldehyde was extracted with ether and the excess ether was removed by aeration. Pyruvate and triose phosphate were determined in different aliquots of the solution. A portion was treated with crystalline triosephosphate dehydrogenase in the presence of DPN, arsenate, and cysteine. From the reduction of DPN, it could be calculated that 0.251 μ mole of triose phosphate had been formed per 0.314 μ mole of pyruvate. This represents an 80 per cent recovery of the theoretical amount of triose phosphate necessary to give a 1:1 ratio. In a parallel experiment, in which authentic D-glyceraldehyde 3-phosphate was incubated with the same enzyme, the same procedure gave a recovery of 78 per cent.

DISCUSSION

The sequence of the initial enzymatic steps involved in the oxidation of galactose by *P. saccharophila* is summarized in Fig. 1. Reaction 1 resembles the dehydrogenation of L-arabinose to L-arabono- γ -lactone, which has been described for the same organism. While the same enzyme might catalyze both oxidations, there is good evidence that at least two different enzymes are produced when the cells are grown with the two substrates². Presumably, the furanose forms of the sugars are converted to the γ -lac-

² Doudoroff, M., and Contopoulou, R., unpublished.

tones The galactose dehydrogenase activity is inducible and is not present in cells grown with sucrose or D-arabinose As shown in Reaction 2, the γ -lactone is rapidly hydrolyzed by an enzyme, which may be identical with the delactonizing enzyme which hydrolyzes L-arabono- γ -lactone Preliminary experiments have shown that this enzyme is also an inducible enzyme In Reaction 3, galactonic acid is dehydrated to 2-keto-

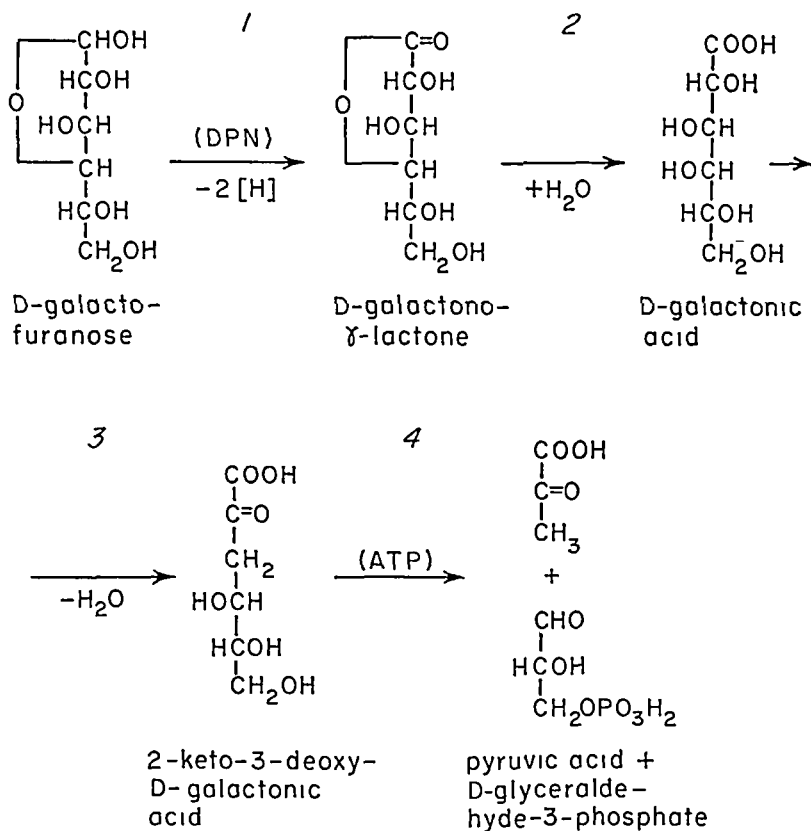


FIG 1 Oxidation of D-galactose by *P. saccharophila*

3-deoxygalactonic acid The equilibrium is far to the right This reaction is analogous to the dehydration of 6-phosphogluconic acid and of D-arabonic acid by the same organism (2, 5) The three enzymes are distinct and the enzyme responsible for Reaction 3 will be designated galactonic dehydrase This enzyme, unlike 6-phosphogluconic dehydrase, is not strongly inhibited by fluoride and is not present in cells grown with sucrose, D-xylose, or D-arabinose Reaction 4 consists probably of at least two steps In the first step, 2-keto-3-deoxy-6-phosphogalactonate is the most likely product of phosphorylation The fact that 2-keto-3-deoxygluconic acid is not decomposed by the same preparations proves that no

Walden inversion at C₄ occurs before the phosphorylation. At present, it is impossible to decide whether or not the phosphorylated keto acid derivatives of gluconic and galactonic acids are interconvertible and whether only one or both of them can serve as a substrate for the ultimate aldolase reaction in which pyruvic acid and triose phosphate are produced. The purifications of the hypothetical 2-keto-3-deoxy-6-phosphogalactonic acid and of the aldolase system are prerequisites to the solution of this problem.

It is interesting that 6-phosphogalactonate, which is not the normal intermediate, is slowly split by the crude enzyme preparations. Since this decomposition is inhibited by fluoride, it seems likely that the compound is either slowly converted to 6-phosphogluconate or dehydrated directly by 6-phosphogluconic dehydrase, rather than attacked by the galactonic dehydrase described in the present paper.

SUMMARY

The following reactions are carried out by cell-free extracts of *Pseudomonas saccharophila* grown with galactose: (1) a diphosphopyridine nucleotide-linked oxidation of galactose to galactono- γ -lactone, (2) the hydrolysis of the lactone to galactonic acid, (3) the dehydration of galactonic acid to 2-keto-3-deoxygalactonic acid, (4) the reaction of the keto acid with adenosine triphosphate to yield pyruvic acid and D-glyceraldehyde 3-phosphate.

2-Keto-3-deoxygalactonic acid has been prepared enzymatically and crystallized as the potassium salt. It has also been synthesized chemically from metasaccharin. The glucose analogue has been synthesized and found to be enzymatically inactive. 6-Phosphogalactonic acid is attacked at a low rate by crude enzyme preparations, but does not appear to be a normal intermediate in galactose metabolism.

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AN UNIDENTIFIED IODINE COMPOUND FORMED BY INCUBATION OF CELL-FREE PREPARATIONS OF TISSUE WITH IODIDE- I^{131} *

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In a previous communication (1), we described the formation of a mono-iodotyrosine-containing protein by various subcellular fractions of thyroid tissue incubated *in vitro* with iodide- I^{131} . The most active fraction was that containing the mitochondria and microsomes, and this fraction formed, in addition to I^{131} protein, an unidentified I^{131} component which moved with the solvent front during filter paper chromatography. This component is designated in our laboratory as Unknown 1.

More recently, we encountered another unknown I^{131} component on our filter paper chromatograms, which, in some cases, comprises more than 75 per cent of the I^{131} added to the incubation medium. This component, which we refer to as Unknown 2, does not separate from iodide when collidine-water-ammonia is used as the chromatographing solvent, and it was therefore not observed in our earlier work in which this solvent was the only one employed. Unknown 2 was first observed when we began to use butanol-ethanol- NH_4OH as a chromatographing solvent, in addition to collidine. The presence of the new component was then confirmed in other solvent systems, as described below. By testing other animal tissues and their cellular fractions, we discovered that homogenates and extracts of rat submaxillary tissue are even more active than are thyroid preparations in forming Unknown 2. Particulate fractions of mammary and spleen tissue are also quite active.

In this communication, we describe the results of initial experiments designed to determine the nature of Unknown 2 and the factors which affect its formation.

Methods

Sheep thyroids were obtained at a local abattoir and transported to the laboratory in a Dewar flask packed with crushed ice. Other tissues were obtained from Long-Evans rats, raised and maintained on Purina laboratory chow.

Homogenization of Tissue—All tissues were homogenized in a specially

* Aided by a grant from the United States Public Health Service

constructed, stainless steel tissue press, designed to provide cellular disruption with a minimum of damage to cell nuclei (2) Tissues were chilled in an ice bath and minced with scissors before being transferred to the tissue press 7 gm of tissue were homogenized with 35 ml of Krebs Ringer-bicarbonate buffer at pH 7.4

Isolation of Subcellular Fractions—Differential centrifugation was used to separate the tissue homogenates into the following (1) a nuclear fraction, (2) a mitochondrial-microsomal fraction, and (3) a soluble fraction The procedure employed was similar to that described previously (1) Most of the experiments were concerned with the mitochondrial-microsomal fraction, referred to here as the "particulate" fraction

The sedimented particulate and nuclear fractions were suspended in cold Krebs-Ringer-bicarbonate buffer at pH 7.4 prior to incubation with I^{131} Approximately 1.7 ml of buffer were added for each gm of original tissue, and a uniform suspension was attained by dispersing the material in the buffer with the aid of a Teflon glass homogenizer

Incubation with I^{131} —2 ml portions of the whole homogenates or of the subcellular fractions were incubated in 10 ml glass-stoppered Erlenmeyer flasks with I^{131} -iodide (10 to 20 μ c, purified by distillation) In some experiments, carrier iodide was added at a concentration of 10 or 25 γ per cent Inhibitors, when present, were added in 20 μ l of solution The air in the reaction flasks was replaced with 95 per cent O_2 -5 per cent CO_2 , and the flasks were shaken in a constant temperature bath at 37° for 1 or 2 hours¹ When anaerobic conditions were desired, the tissue homogenate and its subcellular fractions were suspended in Krebs-Ringer-bicarbonate buffer which had been saturated with 95 per cent N_2 -5 per cent CO_2 , and the air in the reaction flasks was replaced with the same gas mixture

As each incubation flask was removed from the constant temperature bath, it was cooled in an ice bath, and 20 μ l of 1 per cent thiouracil were added to stop iodination reactions 20 μ l of the flask contents were then analyzed directly by filter paper chromatography The presence of a small concentration of thiouracil (8×10^{-4} M) in the mixture to be chromatographed minimized the occurrence of chromatographic artifacts (1)

Chromatographic Analysis—The filter paper chromatographic procedures have been described previously (1, 3, 4) All solvents used for chromatography were purified by distillation Freshly prepared chromatographing solvent was used for the preparation of all chromatograms The I^{131} in the various components was determined by cutting out the zones of

¹ When cyanide was used as an inhibitor, it was added to the reaction flask after the air in the reaction vessels had been replaced with 95 per cent O_2 -5 per cent CO_2 This was done to avoid loss of cyanide (as HCN) during the gas flushing procedure

activity on the chromatograms and counting them directly in a well type scintillation counter. The radioactivity in each component was then expressed as a percentage of the total I^{131} on the chromatogram. This calculation neglected the small amount of radioactivity (<5 per cent of the total) which was present in the areas between those components visible on the radioautographs.

*Experiments with Sheep Thyroid Tissue Slices and Rat and Mouse Thyroids Labeled *in Vivo**—A few experiments were performed in which the chromatographic techniques described above were applied to (1) sheep thyroid tissue slices incubated in Kiebs-Ringer-bicarbonate buffer containing I^{131} -iodide, and (2) rat and mouse thyroids labeled *in vitro* with I^{131} .

200 mg. of slices were incubated in 2 ml. of buffer for 1 to 2 hours. After the incubation, the slices were separated by filtration, rinsed briefly in non-radioactive Ringer's solution, and homogenized with 1 ml. of bicarbonate buffer containing approximately 0.001 M thiouracil. 20 μ l. of the homogenate were applied to filter paper strips for chromatographic analysis.

Thyroids were removed from rats and mice that had been injected with I^{131} 1 to 7 hours previously. In some of the animals, organic I^{131} formation was blocked by a single injection of propylthiouracil. The thyroid tissue was homogenized with bicarbonate buffer containing approximately 0.001 M thiouracil, 0.5 ml. being used for the rat thyroids and 200 μ l. for the pooled glands from two mice. 20 μ l. of the homogenates were used for filter paper chromatography. The submaxillary glands in some of the mice were also homogenized with bicarbonate buffer and analyzed by filter paper chromatography.

Results

Use of Different Chromatographing Solvents for Separation of Unknown 2—Fig. 1 shows a series of radioautographs of chromatograms prepared from a sheep thyroid particulate fraction that had been incubated for 2 hours with iodide- I^{131} (no I^{127} carrier added). A different chromatographing solvent was used for the preparation of each chromatogram. Fig. 1, a, shows the results obtained with the collidine-water-ammonia solvent. The distribution of I^{131} on the chromatogram agrees essentially with that reported previously (1), namely, (1) a band for I^{131} protein at the origin, (2) an unidentified band at the solvent front (Unknown 1), and (3) a band for iodide.

Fig. 1, b to d, shows the results obtained with other chromatographing solvents. These include (1) butanol-ethanol-2 N NH_4OH , 100:20:40, (2) butanol-ethanol-water, 100:20:40, and (3) propanol-0.15 N NH_4OH ,

100 50 In all of these solvents, the I^{131} , which in the collidine-water- NH_3 chromatography system appears to be a single band for iodide, is split into two bands. These same two bands were observed when the iodide band of the collidine chromatogram was cut out and rechromatographed in butanol-ethanol-2 N NH_4OH . Control experiments established that iodide alone gives only a single band in the solvents used for Fig 1,

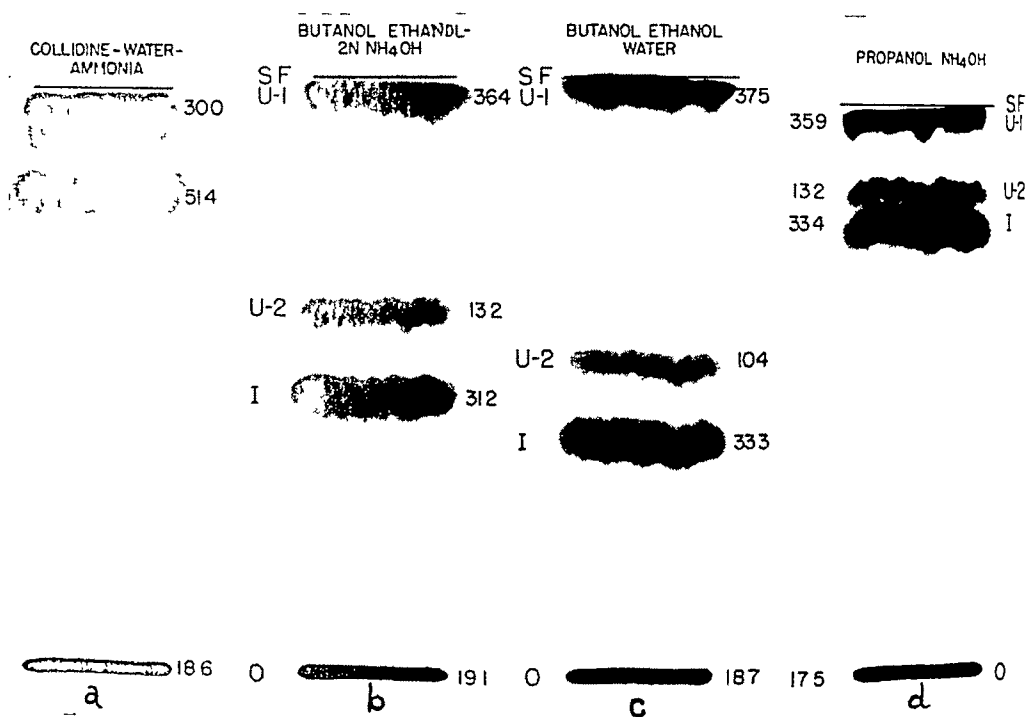


FIG 1 Radioautographs of chromatograms of a sheep thyroid particulate fraction that had been incubated for 2 hours with I^{131} -iodide. A different chromatographing solvent was used in each case, as indicated. The numbers alongside the bands denote the percentage of the total I^{131} on the paper present in the different components. O, origin, I, iodide, U-1, Unknown 1, U-2, Unknown 2, S F, solvent front.

b to d. There can be little doubt, therefore, that a previously unrecognized I^{131} component is formed during the incubation of the thyroid particulate fraction with I^{131} , and we have named this new component "Unknown 2."

The quantitative distribution of I^{131} on the chromatograms is indicated in Fig 1 by the numbers which appear beside each band. There is fairly good quantitative agreement among the results obtained with different chromatographing solvents, not only for Unknown 2, but also for the other I^{131} components on the chromatogram.

The percentage of added I^{131} (carrier-free) converted to Unknown 2

by thyroid particulate fractions varied considerably from one experiment to another and appeared to be inversely related to the formation of I^{131} protein. In some cases, Unknown 2 comprised more than 75 per cent of the total I^{131} on the chromatogram, but it was usually less than 25 per cent. With a given sample of thyroid tissue, the formation of Unknown 2 was quantitatively reproducible.

Formation of Unknown 2 by Thyroid Nuclear and Soluble Fractions, by Thyroid Tissue Slices, and by Intact Thyroids—Whole thyroid homogenates displayed variable activity in forming Unknown 2 from added iodide. In some experiments, more than 25 per cent of the added I^{131} was converted to Unknown 2 by sheep thyroid homogenates, but usually the conversion was much less. The nuclear fraction of thyroid tissue was relatively inactive, and the soluble fraction was completely inactive. All of the activity of the whole homogenate, therefore, could be accounted for by the particulate fraction.

When sheep thyroid tissue slices were incubated *in vitro* with iodide- I^{131} , only a very small fraction of the added I^{131} (less than 2 per cent) was converted to Unknown 2. Rat or mouse thyroids, labeled *in vitro* with I^{131} , did not contain any detectable Unknown 2. This was true both for propylthiouracil-blocked and -unblocked glands. Moreover, Unknown 2 could not be detected in mouse submaxillary tissue, in which, in some instances, the concentration of iodide- I^{131} was more than 20 times that of the plasma level.

Formation of Unknown 2 in Non-Thyroid Tissues—Whole homogenates and subcellular fractions of several non-thyroid tissues were tested for their ability to form Unknown 2. These tissues, all obtained from adult rats, included submaxillary, lactating mammary, spleen, liver, and kidney. The results obtained with the particulate fractions are shown in Fig. 2. Rat submaxillary was the most active, and kidney the least active, of all the tissues tested.

It is of particular interest that the soluble fraction of submaxillary was even more active than the particulate fraction in forming Unknown 2. The soluble fraction of all other tissues, including the thyroid, was completely inactive.

Formation of Unknown 2 As Function of Time—Time curves for the formation of Unknown 2 in thyroid and in salivary tissue are shown in Fig. 3. Two separate curves are illustrated for thyroid particulate fractions. One curve was obtained with an extremely active preparation, the other with a moderately inactive preparation.

Curves for both the particulate and the soluble fractions of submaxillary tissue are also shown in Fig. 3. The activity displayed by these fractions is typical of the results of several different experiments.

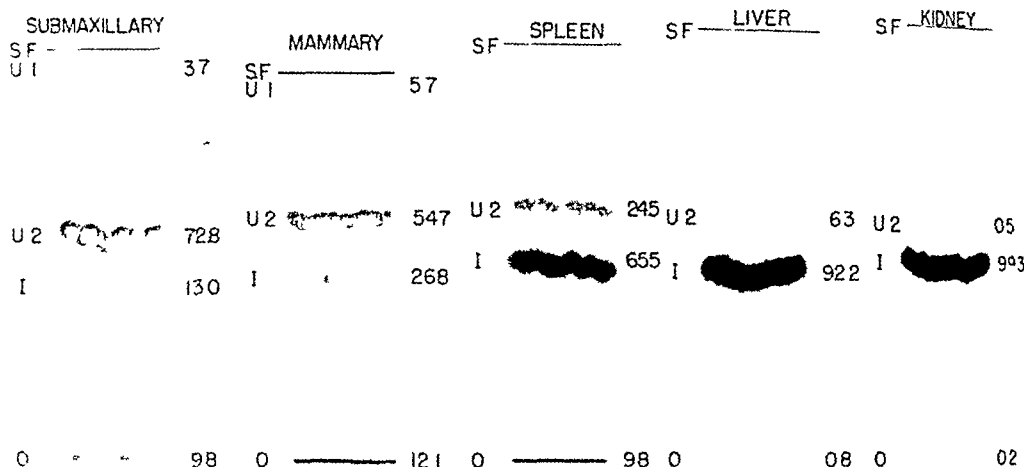


FIG 2 Radioautographs of chromatograms of particulate cell fractions that had been incubated with carrier-free I^{131} -iodide for 2 hours. The particulate fractions were derived from various rat tissues, as indicated. Solvent, butanol-ethanol 2:1 NH_4OH . See Fig 1, legend, for an explanation of numbers and symbols.

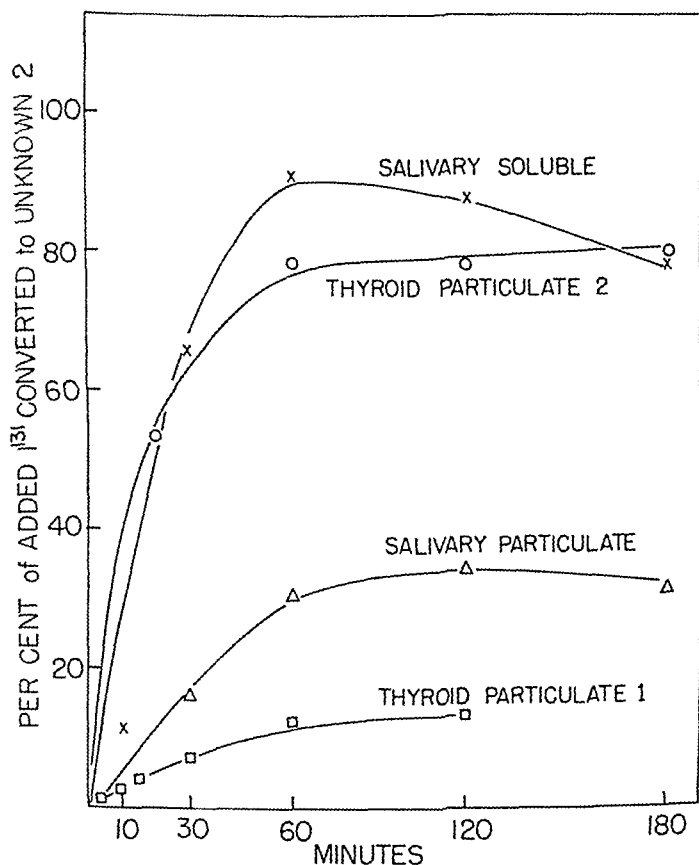


FIG 3 Time curves showing formation of Unknown 2 by subcellular fractions of tissue incubated with I^{131} . No iodide carrier was added to the thyroid particulate fractions, but 25 γ per cent of iodide were added to the salivary fractions. The two thyroid preparations were derived from different groups of sheep and illustrate extremes of activity.

Effect of Inhibitors on Formation of Unknown 2—The results in Table I demonstrate that the formation of Unknown 2 is readily inhibited by the same agents which interfere with organic iodine formation in thyroid tissue slices (5, 6) Thiouracil, thiocyanate, thiosulfate, and cyanide were all markedly inhibitory, and anaerobic conditions, too, had a definite inhibitory effect. Since all of these agents are known to interfere with the

TABLE I
Effect of Inhibitors on Formation of Unknown 2

Tissue preparation	Incubation time	Treatment	Iodide carrier added to incubation medium	Formation of Unknown 2 as per cent of control	Control value for Unknown 2 formation
	hrs		γ per 100 ml		per cent of total I^{131}
Thyroid particulate	1	N ₂ atmosphere	0	24	14.7
“ “	2	“ “	10	27	14.6
“ “	2	10 ⁻³ M thiouracil	10	2	14.6
“ “	2	10 ⁻³ M thiocyanate	10	4	14.6
“ “	2	10 ⁻³ M thiosulfate	10	3	14.6
“ “	2	10 ⁻³ “ cyanide	10	5	14.6
“ “	2	Heated at 100° for 1.5 min	10	5	14.6
Salivary “	2	10 ⁻³ M thiouracil	0	0	72.8
“ “	2	Heated at 100° for 1 min	25	0.3	34.6
“ soluble	1	N ₂ atmosphere	0	13	76.4
“ “	2	Heated at 100° for 1 min	25	0.2	81.4
Lactating mammary particulate	2	N ₂ atmosphere	0	87	53.1
“ “	1	“ “	500	38	9.0
“ “	2	10 ⁻³ M thiouracil	0	0	53.1
“ “	2	Heated at 100° for 5 min	0	0	54.7

oxidation of iodide, it appears reasonable to conclude that oxidation of iodide is a necessary preliminary step in the formation of Unknown 2.

Tissue preparations which were heated at 100° for 1 to 2 minutes completely lost their ability to form Unknown 2 (Table I). This indicates that tissue enzymes are most likely involved in the formation of this unknown compound.

Elution of Unknown 2 from Filter Paper Chromatograms—Samples of I¹³¹-labeled Unknown 2 were obtained by cutting out the zones of the chromatogram corresponding to this compound and eluting it from the

paper Elution with isotonic NaHCO_3 was quantitative and yielded a solution which, when rechromatographed, showed that the compound had not been essentially altered However, inorganic iodide, amounting to 10 to 20 per cent of the total I^{131} , was usually present in the eluates The presence of iodide in the eluates suggested that Unknown 2 is quite labile Other elution solvents were tried, but none proved more satisfactory than NaHCO_3

Experiments with Eluates of Unknown 2—Since only minute quantities of Unknown 2 were available, it was necessary to depend entirely upon chromatography to determine the effect of various agents on this compound Eluates of the compound were treated in various ways and then rechromatographed to determine the effect of the treatment An untreated sample of the eluate was always chromatographed for comparison

It appeared that Unknown 2 might simply be an oxidized form of inorganic iodine, and the first experiments consisted of attempts to reduce the compound to iodide with various reducing agents As shown in Table II, reducing agents such as thiosulfate, sulfite, arsenite, and thiouracil had very little effect on Unknown 2, even after incubation for 2 hours at 37° in bicarbonate solution

The effects of acid and alkali were tried next, and it was observed that Unknown 2 is extremely labile in acid, even at pH 5 As shown in Table II, treatment at pH 5 for only a few minutes resulted in almost complete destruction of the compound I^{131} -iodide was the only radioactive product formed² Treatment with strong alkali (approximately 0.3 N NaOH), on the other hand, had no effect on Unknown 2

The effect of pH on Unknown 2 is illustrated in Fig. 4 The data were obtained by diluting samples of Unknown 2 eluate with buffers (10 volumes) of different pH Each diluted sample was incubated for 1 hour at 37° and then rechromatographed, after the addition of alkali The amount of Unknown 2 remaining was compared with that present in the untreated eluate It is apparent from Fig. 4 that Unknown 2 is relatively stable above pH 7, but that below pH 5 its I^{131} is readily converted to inorganic iodide

When eluates of Unknown 2 were heated at 100° for an hour, approximately 50 per cent of the I^{131} was converted to inorganic iodide (Table II) The compound is, therefore, fairly heat-labile

Inorganic iodide in high concentration (12.5 mg per cent) was added to an eluate of Unknown 2 to determine whether there was any exchange

² It should be pointed out that elemental iodine (I_2^{131}) would also have appeared as iodide on our filter paper chromatograms, since the solvent used does not separate iodide from iodine However, the presence of I_2^{131} appears to be excluded by the extraction results described below

TABLE II
Treatment of Unknown 2 with Various Agents

Tissue source of eluate	Treatment	Per cent of initial Unknown 2 remaining
Thyroid particulate	5×10^{-3} M thiouracil, 37°, 2 hrs	94
" "	5×10^{-3} " thiosulfate, 37°, 2 hrs	96
" "	5×10^{-3} " sulfite, 37°, 2 hrs	93
" "	12.5 mg % iodide, 37°, 2 hrs	100
" "	pH 5.3, 37°, 10 min	2
" "	" 1.6, 20°, 10 "	2
" "	0.3 N NaOH, 37°, 2 hrs	100
" "	Heated 100° for 1 hr	37
Salivary	5×10^{-3} M thiouracil, 37°, 2 hrs	94
" "	5×10^{-3} " thiosulfate, 37°, 2 hrs	92
" "	5×10^{-3} " arsenite, 37°, 2 hrs	98
" "	12.5 mg % iodide, 37°, 2 hrs	68
" "	pH 4.4, 20°, 2 min	5
" "	" 1.6, 20°, 2 "	5
" "	0.3 N NaOH, 37°, 2 hrs	99
" "	Heated 100° for 1 hr	43
" "	Pancreatin (10 mg per ml), 37°, 24 hrs	92
" "	1.5 % H_2O_2 , 37°, 1.5 hrs	32

Eluates of Unknown 2 from paper chromatograms were treated with various agents in bicarbonate solution and then rechromatographed

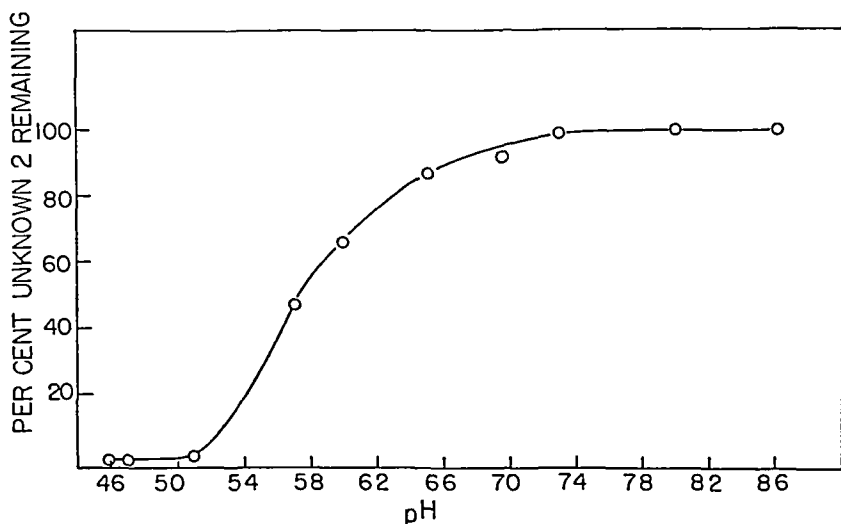


FIG 4 Effect of pH on eluate of Unknown 2. Portions of the eluate were diluted with 10 volumes of buffer, incubated for 1 hour at 37°, alkalinized, and then rechromatographed to determine what fraction of the original Unknown 2 remained

between iodide and the I^{131} in the unknown compound. The concentration of stable iodine in the unknown, although not determined, was probably much less than 0.1 per cent of that of the added iodide, thus favoring the conversion of I^{131} to iodide by exchange. As shown in Table II, the results were variable. In one experiment, no exchange occurred, but, in another, there was a small degree of exchange. In no case, however, was any large degree of exchange observed.

The addition of pancreatin (10 mg per ml) to an eluate of Unknown 2 had very little effect (Table II). However, pancreatin did have a pronounced effect when added to a tissue preparation containing Unknown 2. Most of the I^{131} present as Unknown 2 in an incubated thyroid particulate fraction was converted to inorganic I^{131} after incubation of the sample with pancreatin. The deiodinating action of pancreatin under the latter conditions was not further investigated.

Treatment of an Unknown 2 eluate with 1.5 per cent hydrogen peroxide (in bicarbonate solution) converted an appreciable part of the I^{131} (20 per cent) to iodate. The latter appeared as a band immediately above the origin (R_F 0.05 in butanol-ethanol-2 N NH_4OH). There was also a heavy band for I^{131} -iodide on the chromatogram. Only 32 per cent of the original Unknown 2 remained after 1.5 hours of incubation at 37° (Table II).

Extractability of Unknown 2—Eluates of Unknown 2, both in bicarbonate solution and in acid solution, were shaken with equal volumes of chloroform or carbon tetrachloride. Distribution ratios were determined by counting the I^{131} in the aqueous and organic phases. The I^{131} in Unknown 2 was not extracted by these solvents, even from acid solution. This finding appears to exclude the possibility that the I^{131} in Unknown 2 is present in the form of elemental iodine (i.e. an I_2 complex).

Distribution tests were also made with butanol as the organic solvent. In this case, the distribution ratio was 5.1 in favor of the aqueous phase. I^{131} -iodide under the same conditions ($NaHCO_3$ solution) gave a distribution ratio of 9.1 in favor of the aqueous phase.

DISCUSSION

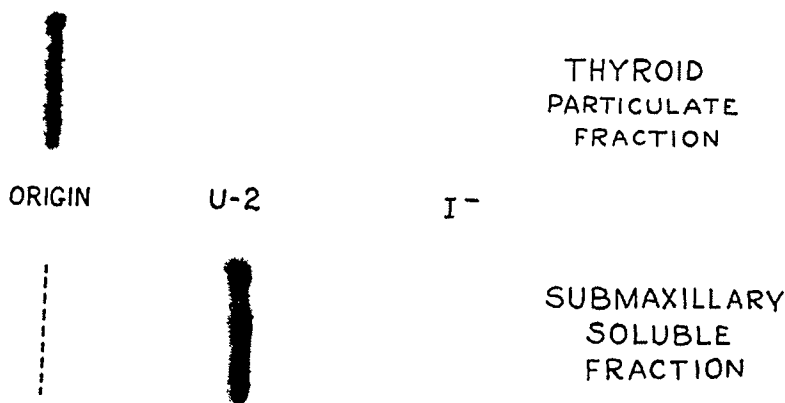
The unidentified I^{131} component (Unknown 2) described in this communication is not an artifact of chromatography. It has been detected with many different chromatographing solvents, both neutral and ammoniacal. The neutral solvents included butanol-ethanol-water, butanol-dioxane-water, butanol-water, and propanol-water. The ammoniacal solvents included butanol-ethanol- NH_4OH , butanol-dioxane- NH_4OH , propanol- NH_4OH , and butanol-pyridine-water in an ammonia atmosphere. Unknown 2 has also been detected by paper electrophoresis (Fig. 5).

We have assumed that the same unidentified I^{131} component is formed

by the particulate fraction of all the different tissues studied here because this component has nearly the same R_F value (in butanol-ethanol-2 N NH_4OH) no matter what its source. Furthermore, in the case of thyroid and salivary tissue, the behavior of eluates of the unknown compound toward various agents is essentially the same.

The following evidence indicates that the formation of Unknown 2 by homogenates and subcellular fractions of tissue depends on tissue enzymes:

(1) Otherwise active tissue preparations, exposed to a temperature of 100°



PAPER ELECTROPHORESIS PATTERN

FIG 5 Radioautograph of paper electrophoretogram showing distribution of I^{131} in thyroid particulate and submaxillary soluble fractions incubated for 90 minutes with I^{131} -iodide. Unknown 1 and I^{131} -protein remained at the origin. Conditions of electrophoresis: Whatman No 1 filter paper, 0.05 M Veronal buffer, pH 8.6, 495 v, 5 ma, 60 minutes duration.

for 1 minute, were completely inactive. (2) No Unknown 2 appeared in tissue preparations to which I^{131} was added immediately after, instead of before, incubation.

Tissue enzymes concerned with oxidation appear to be involved in the formation of Unknown 2. This is indicated by the nature of the agents and conditions which inhibit the formation of this compound. Anaerobic conditions and the presence of 10^{-3} M cyanide were inhibitory. Also, the reaction is inhibited by those agents which are known to interfere with the oxidation of iodide by intact thyroid tissue, *e.g.* thiouracil and thio-sulfate. The oxidative step involved in the formation of Unknown 2 is most likely required for the oxidation of iodide, since otherwise it is necessary to conclude that some other compound is oxidized and is then

able to bind inorganic iodide in some manner. The latter possibility appears to be excluded by the finding (Table II) that the I^{131} in Unknown 2 does not exchange with a large excess of inorganic iodide.

Although the identity of Unknown 2 remains to be determined, the following statements regarding its chemical nature appear to be justified at this time. (1) It is not simply an oxidized form of inorganic iodine, i.e. I_2 , hypoiodite, or iodate. Elemental iodine (I_2) and hypoiodite are converted to inorganic iodide by treatment with thiouracil or thiosulfate in bicarbonate solution, whereas Unknown 2 is unaffected under these conditions (Table II). Moreover, the R_F values (in butanol-ethanol-2 N NH_4OH) of hypoiodite³ (0) and iodate (0.05) do not agree with that of Unknown 2 (0.5 to 0.6). (2) The fact that Unknown 2 is so readily converted to inorganic iodide by weak acid (pH 5) almost certainly rules out the possibility that it contains a C—I bond. The finding that it is so labile in acid, yet stable in alkali, would also seem to rule out a sulfonyl iodide. Compounds of the latter type, recently proposed by Fraenkel-Conrat as possible intermediates in the iodination of protein (7), would be expected to be even more unstable in alkali than in acid. (3) Unknown 2 does not appear to be a complex involving elemental iodine, since its I^{131} is not extractable with chloroform, even after acidification.

Zingaro *et al.* (8) studied the reaction between elemental iodine (I_2) and the heterocyclic amines, pyridine and quinoline. They postulated that a reaction of the following type may occur: $Pyridine + I_2 \rightarrow Pyridine I^+ + \bar{I}$. The complex, $Pyridine I^+$ ($Py I^+$), supposedly contains unipositive iodine (I^+) attached to the ring nitrogen. This group of investigators also described a series of compounds (9) in which unipositive iodine is apparently stabilized by coordination with pyridine or quinoline, compounds of the type $PyI^+\bar{X}$ and $QuI^+\bar{X}$. Such compounds might be expected to be particularly unstable in acid because of the competition between hydrogen ions and I^+ for the free pair of electrons on the nitrogen. The striking acid instability of Unknown 2 leads us to suggest that it, too, may contain unipositive iodine attached to the nitrogen of one of the various organic bases known to be present in tissue. However, it is evident from Fig. 5

³ A solution containing I^{131} -hypoiodite was prepared as follows. I^{131} -iodide was oxidized with iodate in acid solution, and the I_2^{131} which was produced was extracted into chloroform. The chloroform solution was washed with water and then shaken with 0.15 M $NaHCO_3$. The $NaHCO_3$ extract, which contained about two thirds of the original I^{131} , presumably contained part of its I^{131} as hypoiodite, according to the reaction $I_2 + H_2O \rightarrow HI + HIO$. Chromatography of the $NaHCO_3$ extract revealed bands for iodide and iodate and, in addition, a band at the origin. The latter was not present if the $NaHCO_3$ solution was first treated with thiouracil, and it was assumed to be due to hypoiodite. The iodate most likely arose from hypoiodite via the reaction $3HIO \rightarrow IO_3 + 2\bar{I}$.

that at pH 8.6 Unknown 2 behaves as an anion, moving in the same direction as iodide. If indeed, therefore, Unknown 2 contains positively charged iodine attached to nitrogen, then this must be counteracted by acidic groups in the rest of the molecule to make it behave as an anion.

The biological significance of Unknown 2, if any, remains to be determined. It does not appear to be involved in the normal iodine metabolism of the thyroid, since it is not observed in the thyroids of rats or mice injected with I^{131} . Its formation in the thyroid particulate fraction appears to be inversely related to the formation of I^{131} protein, thus suggesting a competitive reaction rather than an intermediate step in the iodination of tyrosine. It is not involved in the iodide trap since it is not observed in propylthiouracil-blocked rat or mouse thyroids, or in mouse salivary glands, all of which actively concentrate inorganic iodide.

The assistance of Dr. G. D. Potter in the early phases of this work is gratefully acknowledged.

SUMMARY

1. Whole homogenates and subcellular fractions of thyroid, submaxillary, mammary, spleen, liver, and kidney tissue were incubated with I^{131} iodide. The nature of the I^{131} compounds formed during the incubation was investigated by filter paper chromatography.

2. The particulate fraction (mitochondria and microsomes) of all tissues, except kidney, converted an appreciable part of the added I^{131} to an unknown I^{131} -containing compound, designated here Unknown 2.

3. The soluble fraction of salivary tissue was the most active preparation tested, converting as much as 90 per cent of the added I^{131} to Unknown 2 in 1 hour. The soluble fraction of all other tissues was inactive.

4. The formation of Unknown 2 depends upon tissue enzymes. The reaction is markedly inhibited by anaerobic conditions, as well as by thiouracil and thiosulfate, indicating that oxidative enzymes are involved.

5. Very dilute samples of Unknown 2 were obtained by eluting the appropriate sections of filter paper chromatograms with sodium bicarbonate solution. Tests carried out on such eluates revealed that Unknown 2 is rapidly destroyed in acid solution, even at pH 5.0. In alkaline solution, however, it is stable. Results of other chemical tests are also reported.

6. The chemical findings are discussed with reference to possible structures for Unknown 2.

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ACTIVATION OF THE IODINATING SYSTEM IN SHEEP THYROID PARTICULATE FRACTIONS BY FLAVIN COFACTORS*

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In an earlier report (1), we described the preparation, from sheep thyroid glands, of a particulate fraction consisting primarily of mitochondria and microsomes, which, when incubated with carrier-free radioactive iodide, converted as much as 30 per cent of the added I^{131} to labeled iodoprotein. This thyroid particulate fraction differed from the cell-free thyroid preparations used by other workers (2, 3) in the following respects: (1) it was active in the absence of added tyrosine and oxidizing agents, and (2) the major product formed was iodoprotein, not free iodotyrosine.

In experiments in which the iodine metabolism of the particulate fraction was compared with that of thyroid slices, it was observed that the particulate preparation had a much lower capacity to utilize added carrier iodide, moreover, iodination by the particulate fraction led to the formation of an iodinated protein containing I^{131} almost exclusively in the form of moniodotyrosine, whereas the iodinated protein formed by the slice contained almost as much I^{131} -diiodotyrosine as I^{131} -moniodotyrosine. The present report deals with our attempts to enhance the iodinating activity of the thyroid particulate fraction. It was found that the capacity of the particulate system to convert added iodide to iodoprotein could be greatly increased by adding flavin cofactors or by increasing the pH of the incubation medium, but in both instances the newly formed iodoprotein still contained I^{131} almost exclusively in the form of moniodotyrosine.

EXPERIMENTAL

Preparation of Particulate Iodinating System—Fresh sheep thyroid glands were obtained at a local abattoir. Considerable variation was noted in the sizes of the glands, but those selected for study ranged from 1.3 to 1.8 gm per lobe. During the 2 to 3 hours required for transporting them to the laboratory and preparing them for incubation, the glands were kept ice-cold either in crushed ice or in ice-cold buffer solution. The glands were freed of extraneous tissue, minced with scissors, and then

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homogenized in a stainless steel homogenizer devised in this laboratory (4) 7 gm of minced tissue were homogenized with 35 ml of the ice-cold, calcium-free buffer, pH 7.4, described below This homogenate was centrifuged for 10 minutes at $500 \times g$ and at 0° The resulting supernatant liquid was removed and centrifuged at 70,000 to 100,000 $\times g$ in a Spinco model L centrifuge for 30 minutes The sedimented pellet of particulate material was dispersed in 12 ml of buffer with the aid of a Teflon glass homogenizer

Buffer Solutions—The buffer solution used in the homogenization of the thyroid glands and the incubation of the isolated particulate material was a Krebs-Ringer-bicarbonate buffer in which the 0.11 M CaCl_2 solution was replaced by an equal volume of 0.9 per cent NaCl This modification prevented the precipitation of insoluble calcium salts in the experiments carried out under alkaline conditions For the experiments in which the effect of varying pH was studied, the tissue was first homogenized in a buffer of pH 7.4, and the particulate material thus isolated was then suspended and incubated in a buffer at the desired pH

The buffer solutions were gassed with pure O_2 or with a mixture of 95 per cent O_2 and 5 per cent CO_2 For anaerobic experiments, the buffer was gassed with either N_2 or a mixture of 95 per cent N_2 and 5 per cent CO_2 The use of the gas mixtures containing CO_2 had no consistent influence on the activities of the tissue preparations, provided the proper pH adjustments were made before the buffers were used

Incubation Procedures—2 ml portions of the suspensions of particulate material were placed in 10 ml glass-stoppered Erlenmeyer flasks 5 μl of solution containing 2 γ of carrier iodide were added to give a concentration of 100 γ per cent Then 5 to 25 μc of radioiodide, purified by distillation (1), were added in 5 to 10 μl of solution Substrates, inhibitors, and coenzymes¹ were added in 5 to 20 μl of solution The flasks were kept in an ice bath until the incubation mixtures were assembled, they were then placed in a Dubnoff metabolic incubator shaker at 37° and agitated gently for 2 hours At the end of the incubation period, 20 μl of 1 per cent thiouracil solution were added to each flask to stop the reaction Portions of the flask contents were then analyzed directly by paper chromatography, both before and after hydrolysis with enzymes The enzymatic digestion procedure has been described elsewhere (1)

Chromatographic Analysis—The paper chromatographic procedures used here were essentially the same as those described elsewhere (5) Chromatograms of the incubated reaction mixtures were developed mostly

¹ The following abbreviations are used DPN for diphosphopyridine nucleotide, ATP for adenosine triphosphate, FAD for flavin adenine dinucleotide, FMN for flavin mononucleotide

in the butanol-ethanol-2 N NH_4OH solvent mixture of Taurog *et al* (6) Such chromatograms prepared from thyroid particulate material incubated with I^{131} generally showed the presence of four major iodine-containing components (Fig 1) The unidentified components U-1 and U-2 are discussed in the accompanying report (7) The I^{131} -containing material at the origin of the chromatograms is iodinated protein, which may or may not be related to the characteristic thyroglobulin of the thyroid

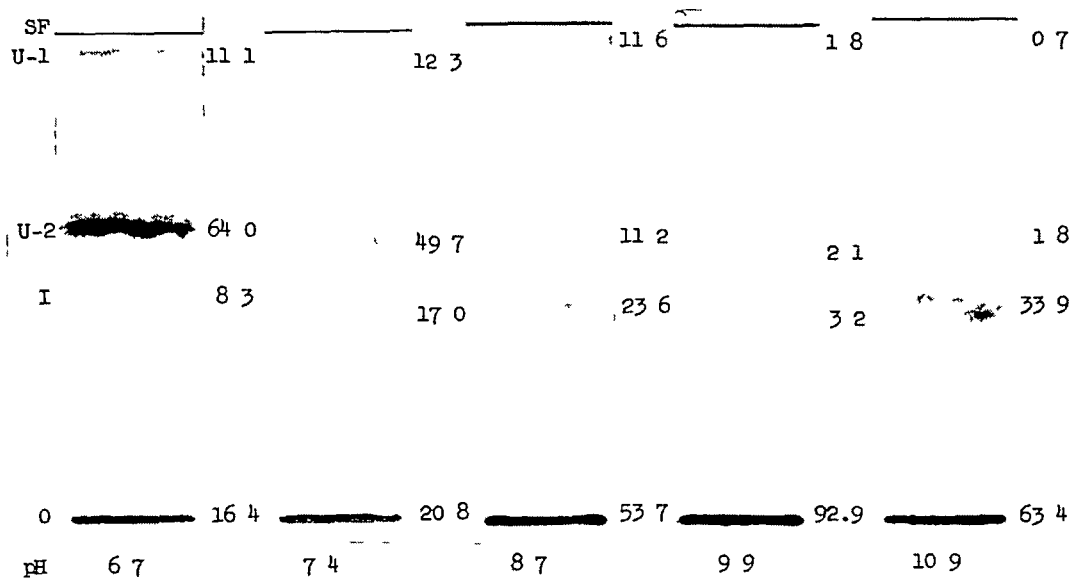


FIG 1 Effect of pH on I^{131} utilization by the sheep thyroid particulate fraction. Radioautographs of chromatograms (butanol ethanol-2 N NH_4OH) prepared from particulate preparations incubated for 2 hours in the presence of 100 γ per cent labeled iodide. The numbers on the right of each radioautograph show the percentages of the total I^{131} on the chromatogram present in the various components. O, origin; I, inorganic iodide; U-2, unidentified component 2; U-1, unidentified component 1; SF, solvent front.

The distribution of radioactivity among the labeled components of a chromatogram was determined by cutting up the chromatograms and assaying the various sections for I^{131} by means of a well type scintillation detector. The results are reported below as percentages of the total I^{131} found on each chromatogram.

Results

Influence of pH of Incubation Medium on Iodide Utilization—The radioautographs of Fig 1 show the effect of varying pH on I^{131} utilization by the sheep thyroid particulate system incubated with 100 γ per cent of

added iodide carrier With ascending pH, increasing amounts of I^{131} were incorporated into protein The maximal incorporation occurred at approximately pH 10

Supplementation of Particulate Iodinating System—In attempts to augment the utilization of radioiodide by the particulate system at physiological pH, the effects of the addition of various coenzymes and substrates were investigated Because previous studies (1) had indicated a requirement for aerobic conditions, the coenzymes and substrates chosen to supplement the particulate fraction were those known to function in respiratory metabolism The results presented in Table I show that the addition of cytochrome *c*, ATP, or the Krebs cycle intermediates, citrate and succinate, did not influence significantly the utilization of radioiodide

TABLE I

Supplementation of Sheep Thyroid Particulate Preparation

2 hour incubations at pH 7.4 in the presence of 100 γ per cent labeled iodide

Material added	Per cent of total I^{131} recovered as			
	Protein	Iodide	U 2	U 1
None	8.2	46.6	35.5	9.9
5×10^{-4} M cytochrome <i>c</i>	7.4	50.9	35.1	6.5
5×10^{-4} " DPN	9.1	12.7	43.0	35.5*
5×10^{-4} " ATP	7.8	43.8	38.4	9.5
5×10^{-4} " cytochrome <i>c</i> + 10^{-2} M succinate	9.5	41.9	39.5	9.1
5×10^{-4} " " " + 10^{-2} " citrate	8.9	46.0	38.0	7.0

* This value includes the I^{131} present in another unidentified component that appeared just below U-1

DPN, on the other hand, did seem to bring about increased radioiodide utilization, however, instead of forming labeled protein, the I^{131} was incorporated into a new component that appeared below U-1 on chromatograms The identity of this component has not yet been established

Activation by Flavin Coenzymes—It was observed that, at pH 7 to 8, the utilization of radioiodide by the particulate system was markedly augmented by the addition of FMN Some of the results are presented in Table II Although there was considerable variation in control values for I^{131} utilization, when the results of each experiment are examined on the basis of the control values for that experiment, it can be seen that even 5×10^{-5} M FMN had definite stimulatory effects on utilization of radioiodide and formation of protein-bound I^{131} Similar results were obtained when the FMN was replaced with either FAD or riboflavin The curve of Fig. 2 shows the effects of increasing concentrations of FMN on the formation of labeled iodoprotein

TABLE II

Flavin Activation of I^{131} Utilization by Sheep Thyroid Particulate Preparation
 2 hour incubations in the presence of 100 γ per cent labeled iodide

Experiment No	pH of incubation	Flavin added	Per cent of total I^{131} recovered as			
			Protein	Iodide	U 2	U-1
1	7.4	None	7.4	52.8	31.0	8.7
		2×10^{-4} M FAD	16.2	21.2	36.8	25.7
		2×10^{-4} " FAD*	0.9	99.1	0	0
		5×10^{-4} " FMN	22.8	16.5	40.5	20.2
		None	5.0	45.4	44.0	5.4
2	7.4	5×10^{-4} M FMN	16.4	16.4	55.4	11.9
		5×10^{-4} " "	15.3	15.3	56.4	12.9
		5×10^{-4} " riboflavin	14.9	17.5	54.4	13.1
		None	12.8	78.1	2.2	6.8
3	8.0	5×10^{-5} M FMN	59.9	10.5	10.7	18.7
		5×10^{-5} " " *	0.6	98.2	1.1	0.1
		None	20.8	17.0	49.7	12.3
4	8.0	5×10^{-5} M FMN	42.5	9.0	32.8	15.7
		5×10^{-5} " " *	1.1	94.9	3.7	0.4
		5×10^{-5} " riboflavin	35.0	9.8	36.7	18.3

* The particulate preparation was immersed in a boiling water bath for 1.5 minutes, then radioiodide and flavin were added for the incubation

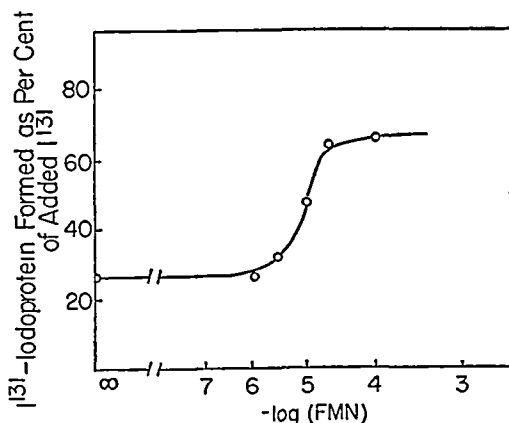


FIG. 2 Influence of varying concentrations of added FMN on the formation of protein-bound I^{131} by the thyroid particulate preparation incubated at pH 8.8 for 2 hours in the presence of 100 γ per cent labeled iodide

Although it seemed unlikely that the flavin compounds could effect a direct oxidation of iodide, the following experiments were conducted to determine whether the iodine utilization by the FMN-supplemented particulate system was enzymatic in nature (1) Samples of the particulate

preparations were adjusted to pH levels of 7.4, 8.5, 9.0, and 10, immersed in boiling water for 15 minutes, and then incubated with 100 γ per cent of labeled iodide and 5×10^{-5} M FMN. (2) Samples of the "soluble fraction" from sheep thyroid tissue (75,000 $\times g$ supernatant extract), containing most of the tissue protein, were incubated with added I^{131} and 5×10^{-5} M FMN. (3) 50 mg portions of a material obtained by dialyzing and then lyophilizing the soluble fraction of thyroid tissue were dissolved in 2 ml portions of buffer at various pH levels and incubated with 100 γ per cent of labeled iodide and 5×10^{-5} M FMN. In no case was a significant utilization of radioiodide observed. Hence it was concluded that the FMN-stimulated formation of organic I^{131} requires a heat-labile, and presumably enzymatic, factor that is present (or active) only in the particulate fraction.

Effects of Various Metabolic Inhibitors—Table III shows the utilization of radioiodide by thyroid particulate preparations, both in the presence and in the absence of FMN when incubated with a number of chemical agents known to have inhibitory effects on various metabolic functions. Not unexpectedly, those chemicals with definite reducing properties, namely thiouracil and cyanide, both had strongly depressing effects on iodide utilization. We have reported previously that these agents also inhibit iodination by a non-enzymatic system consisting of tyrosine, radioiodide, and H_2O_2 (8). Thiocyanate, although not a good reducing agent, also inhibited iodination by both the thyroid particulate and non-enzymatic systems. Perchlorate, which is regarded as having a rather specific inhibitory action on the iodide-concentrating mechanisms of the thyroid gland, had no effect on the particulate iodinating system.

Of the "aminobenzene" type of antithyroid drugs, sulfanilamide had only a slight effect, while both *p*-aminobenzoate and sulfathiazole were strongly inhibitory. *N*-Succinylsulfathiazole, however, was only slightly inhibitory. The latter finding is in agreement with the observation of Taurog *et al* (9) that substitution on the amino groups of sulfanilamide and *p*-aminobenzoate resulted in considerably depressed antithyroid potency in these drugs. It is of interest that chromatograms of the preparations incubated with the succinylsulfathiazole revealed the formation of a new and as yet unidentified iodinated component that appeared in a position immediately below that of the unused iodide. This component accounted for only 5 to 8 per cent of the added I^{131} .

Malonate and monofluoroacetate are believed to block utilization of certain intermediates of the tricarboxylic acid cycle (10), while antimycin A appears to interfere with the electron transport system at some point between the flavins and the cytochromes (11). None of these agents had a pronounced influence on iodide utilization by the particulate iodinating system.

Effect of Catalase—Since it seemed possible that the mechanism of iodide oxidation by a flavin system might involve H_2O_2 , the effect of added catalase enzyme² on radioiodide metabolism by the sheep thyroid particulate preparations was investigated. The results presented in Table IV indicate little, if any, depression of iodide utilization. Indeed, in one experiment

TABLE III

Effects of Various Antithyroid Agents and Metabolic Inhibitors on Radioiodide Metabolism of Sheep Thyroid Particulate Preparation

2 hour incubations at pH 8.0 in the presence of 100 γ per cent labeled iodide

Material added	Per cent of total I^{131} recovered as			
	Protein	Iodide	U 2	U-1
None	23.6	52.4	16.5	7.3
10^{-3} M thiouracil	1.6	98.7	0	0
10^{-3} " KSCN	1.0	99.0	0	0
10^{-3} " $KClO_4$	24.6	51.9	16.9	6.9
10^{-3} " <i>p</i> -aminobenzoate	5.6	89.0	3.7	1.7
10^{-3} " sulfanilamide	26.2	51.2	16.4	6.1
10^{-3} " sulfathiazole	12.2	76.4	8.5	3.0
10^{-3} " succinylsulfathiazole	20.4	60.9	8.3	5.2
10^{-3} " NaCN	0.9	99.1	0	0
10^{-3} " malonate	24.6	53.3	16.2	5.9
10^{-3} " monofluoroacetate	25.3	52.1	16.0	6.6
10^{-3} " antimycin A	26.0	45.1	18.0	10.9
5×10^{-5} M FMN	53.5	13.4	19.2	14.1
5×10^{-5} " " 10^{-3} M thiouracil	2.2	97.8	0	0
5×10^{-5} " " 10^{-3} " KSCN	1.6	98.4	0	0
5×10^{-5} " " 10^{-3} " $KClO_4$	60.9	10.5	14.9	13.7
5×10^{-5} " " 10^{-3} " <i>p</i> -aminobenzoate	7.9	82.8	7.2	2.0
5×10^{-5} " " 10^{-3} " sulfanilamide	42.0	23.4	25.9	8.7
5×10^{-5} " " 10^{-3} " sulfathiazole	14.6	70.9	11.1	3.1
5×10^{-5} " " 10^{-3} " succinylsulfathiazole	31.0	29.2	24.2	7.2
5×10^{-5} " " 10^{-3} " NaCN	0.8	99.2	0	0
5×10^{-5} " " 10^{-3} " malonate	48.6	15.0	22.7	13.7
5×10^{-5} " " 10^{-3} " monofluoroacetate	52.1	13.6	19.9	14.4
5×10^{-5} " " 10^{-3} " antimycin A	41.8	15.6	26.8	15.8

the addition of catalase with FMN at pH 7.5 seemed to enhance the utilization of iodide, resulting in the formation mainly of additional amounts of the unidentified product, U-2. In contrast to this, catalase at concentrations of 0.1 mg per ml completely inhibited radioiodide utilization by a cell-free extract of the marine alga, *Nereocystis*, and by a non-enzymatic iodination system consisting of 10^{-3} M H_2O_2 , 10^{-3} M tyrosine, and I^{131} iodide (8).

² Crystalline catalase, General Biochemicals, Inc.

TABLE IV

*Effect of Catalase on Radiiodide Metabolism of
Sheep Thyroid Particulate Preparation*

2 hour incubations in the presence of 100 γ per cent labeled iodide

Experiment No	pH of incubation	Concentration of FMN	Catalase added	Per cent of total I ¹³¹ recovered as			
				Protein	Iodide	U 2	U 1
1	8 0	None	mg None	13 7	77 6	2 8	5 9
		"	0 1	10 6	82 8	2 2	4 7
		"	0 2	8 6	85 5	1 8	4 2
		"	0 5	5 9	88 7	1 5	3 0
		10 ⁻⁴ M	None	53 7	11 9	19 3	15 0
		10 ⁻⁴ "	0 1	56 7	12 5	17 0	14 1
		10 ⁻⁴ "	0 2	54 6	12 4	18 9	13 9
		10 ⁻⁴ "	0 5	49 8	12 1	25 0	13 0
2	7 5	None	None	7 9	68 6	15 3	8 2
		"	0 1	6 4	73 1	13 7	6 9
		"	0 2	5 1	78 9	10 5	5 4
		"	0 5	3 6	84 7	7 5	4 0
		5 \times 10 ⁻⁴ M	None	20 2	24 2	38 8	16 9
		5 \times 10 ⁻⁴ "	0 1	19 3	18 6	46 9	15 2
		5 \times 10 ⁻⁴ "	0 2	15 8	15 2	55 8	13 0
		5 \times 10 ⁻⁴ "	0 5	11 6	14 8	64 0	9 7

TABLE V

*Effect of Anaerobic Conditions on Utilization of
Radiiodide by Thyroid Particulate System*

2 hour incubations

Experiment No	Atmosphere	pH of incubation	Carrier iodide added	FMN added	Per cent of total I ¹³¹ recovered as			
					Protein	Iodide	U 2	U 1
1	Aerobic	7 8	γ per cent None	None	13 7	18 6	52 4	15 3
			100	"	7 4	52 8	31 0	8 7
			100	5 \times 10 ⁻⁴ M	22 8	16 5	40 5	20 2
	Anaerobic	7 8	None	None	5 3	69 8	18 7	6 2
			100	"	1 6	92 8	4 0	1 5
			100	5 \times 10 ⁻⁴ M	30 9	15 9	22 8	30 3
2	Aerobic	7 9	100	None	13 7	77 6	2 76	5 85
			100	10 ⁻⁴ M	53 8	9 23	23 4	13 5
			100	10 ⁻⁴ M	5 89	89 4	1 1	3 4
	Anaerobic	7 9	None	None	62 8	8 58	11 5	17 0
			100	10 ⁻⁴ M	26 8	53 7	14 3	5 22
			100	10 ⁻⁵ M	46 5	18 1	27 2	8 17
3	Aerobic	8 7	100	10 ⁻⁴ "	65 7	9 65	14 1	10 4
			100	None	22 3	65 5	8 40	3 72
			100	10 ⁻⁵ M	46 5	33 6	12 7	7 13
	Anaerobic	8 8	100	10 ⁻⁴ "	78 4	6 22	6 68	8 72
			100	None	22 3	65 5	8 40	3 72
			100	10 ⁻⁵ M	46 5	33 6	12 7	7 13

Aerobic versus Anaerobic Incubation—It was reported previously (1) that the utilization of radioiodide by the thyroid particulate system was depressed by the exclusion of oxygen. This finding was confirmed in the present investigation, as shown by the data of Table V. However, it was found that the addition of FMN could reverse the inhibitory action of the anaerobic conditions. With greater concentrations of FMN, the requirement for oxygen appeared to diminish, hence the difference between aerobic and anaerobic samples became quite small.

DISCUSSION

The formation of I^{131} protein by the isolated thyroid particulate system can be visualized as occurring in two steps: (1) oxidation of iodide to the I^0 or I^+ states and (2) iodination of the tyrosine residues in the protein. These reactions were not studied separately in the experiments reported here, only the net result of both reactions was observed. Nevertheless, since flavin enzymes are known to function primarily in oxidative processes, we have assumed that the activating action of the added flavin coenzymes occurs primarily at Step 1. Three mechanisms may be suggested for this action: (1) direct oxidation of iodide by the added flavin, (2) activation of a flavin enzyme that oxidizes iodide, or (3) activation of a flavin enzyme that produces H_2O_2 which then oxidizes iodide. Of these three mechanisms, the first is ruled out by the results with boiled preparations (Table II), the second seems unlikely because of the relatively inadequate oxidation-reduction potential of the flavin enzymes, and the third is not supported by our experiments on the effects of catalase and anaerobic conditions on the flavin-stimulated system. In the interpretation of these data, however, it must be borne in mind that iodide utilization by the thyroid gland, as well as by the particulate fraction used here, operates at extremely low levels. For example, Astwood estimates that a 25 gm human thyroid normally oxidizes only 10^{-6} mole of iodide per day (12), and the actual quantity of iodide utilized by 2 ml of the thyroid particulate system after 2 hours of incubation is less than $0.02 \mu\text{mole}$. It is, therefore, conceivable that, with such minute amounts of the substrate, iodide, relatively limited oxidizing systems could perform adequately. Thus, although anaerobic conditions and catalase might be expected to depress H_2O_2 production by a flavin enzyme, sufficient H_2O_2 could remain to oxidize $0.02 \mu\text{mole}$ of iodide, particularly if a peroxidase type enzyme were also present. Such a competition between peroxidase and catalase was suggested by Keston (13) to explain the failure of catalase to inhibit iodination by the xanthine oxidase of milk.

Cyanide is regarded primarily as an inactivator of metalloenzymes, such as cytochromes and peroxidases, while thiocyanate is known for its

inhibitory action on iodide-concentrating mechanisms. However, since both have been found to inhibit iodination by the non-enzymatic (8) as well as by the thyroid particulate system, it is possible that these compounds have some direct chemical influence on the availability of oxidized iodine. It is conceivable, for example, that both cyanide and thiocyanate are capable of blocking the formation of oxidized iodine either by reducing it as it is formed or by reducing the oxidizing agent (*e.g.* H_2O_2) responsible for its formation. Furthermore, both cyanide and thiocyanate are known to form stable compounds or complexes with iodine (14, 15). Although it is best known for its inhibitory action on iodide concentration, thiocyanate has been reported to interfere with the formation of organic iodine by sheep thyroid slices *in vitro* (16), and by a milk xanthine oxidase system (17). The present findings are in agreement with these earlier observations. The fact that perchlorate has no apparent effect on the utilization of iodide by the particulate system is a further indication that the thiocyanate in this system does not act on an iodide-concentrating mechanism.

The increased iodide utilization and iodoprotein formation that occur with increasing pH suggest that increased dissociation of the tyrosyl phenolic groups is involved, since it is known that the iodination of tyrosine proceeds more readily when the phenolic group is ionized (18). Another possibility is that, with increasing pH, the formation of some active form of iodine, such as IO^- , I^+ , or IOH_2^+ (18), may be promoted. On the other hand, it is also possible that the pH optimum of the iodide-oxidizing enzyme itself may lie in the alkaline region.

As reported earlier (1), the I^{131} protein formed by the particulate system contained I^{131} mostly in the form of moniodotyrosine, as revealed by filter paper chromatographic analysis of the enzymatic hydrolysate. Radioactivity in the diiodotyrosine fraction of the hydrolysate usually amounted to only about one-tenth that of the moniodotyrosine fraction. This is in contrast to results obtained with thyroid tissue slices, which usually formed approximately one-half as much I^{131} diiodotyrosine as moniodotyrosine. Predominance of moniodination has been reported in other homogenate systems (3, 19, 20) as well as in incubated slices of cattle thyroids that had been aged at low temperature (21). At first it appeared that the reduced diiodotyrosine formation arose primarily because the iodide-concentrating mechanism had been greatly impaired, thus leading to a reduced concentration of iodide available for iodination of tyrosine residues. However, we found that thyroid tissue slices incubated in the presence of 10^{-3} M perchlorate (which completely blocks the iodide trap) formed I^{131} protein, which, on hydrolysis, yielded mono- and diiodotyrosine in the same proportions as unblocked slices. Moreover,

the thyroids of completely hypophysectomized rats, which concentrated iodide only to about 2 to 3 times the level of plasma, also formed I^{131} -diiodotyrosine very readily (22). These findings suggest that perhaps a special mechanism, not active in tissue homogenates or aged tissue, is required for the conversion of monoiodo- to diiodotyrosine.

Cupric ion was an indispensable component of the homogenate system of Weiss (2), and it has played an important role in the homogenate systems employed by Fawcett and Kirkwood (3). However, the role of copper in the function of the thyroid under physiological conditions has not been clearly defined. The significance of flavin activation in the physiological process of thyroid hormone formation likewise cannot be clearly established at this time. The demonstration by Keston (13), and subsequently by others (17, 23, 24), that xanthine oxidase may catalyze the formation of iodinated protein, suggested the possibility that flavin-containing oxidases might be involved in biological iodination processes. Xanthine oxidase itself, however, has been reported to be absent from the thyroid tissue of a number of mammalian species (25-27). In the light of the present findings with regard to flavin activation of the particulate iodinating system, it becomes of interest to reconsider the possible involvement of the yellow enzymes in thyroid iodine metabolism.

SUMMARY

1 Addition of flavin cofactors markedly stimulated the iodinating system present in mitochondrial-microsomal fractions isolated from sheep thyroid glands. Conversion of added carrier iodide to iodoprotein by such particulate fractions was markedly increased by the presence of 10^{-5} M flavin mononucleotide. Evidence was obtained showing that the stimulatory action of the flavin was enzymatic in nature.

2 The pH optimum for iodide utilization by both the flavin-supplemented and the unsupplemented particulate fraction was between 9 and 10.

3 The stimulating action of flavin on the iodinating system of the thyroid particulate fraction was inhibited by thiouracil, thiocyanate, cyanide, and certain aromatic goitrogens, but not by perchlorate, malonate, mono-fluoroacetate, antimycin A, or catalase.

4 The possibility of the physiological participation of flavin in thyroid iodine metabolism is discussed.

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STIMULATORY EFFECT OF BARBITAL ON URINARY EXCRETION OF L-ASCORBIC ACID AND NON-CONJUGATED D-GLUCURONIC ACID

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When administered to rats, various drugs of widely different chemical structures, for example barbiturates, Chloretone, and paraldehyde, markedly increase the urinary excretion of L-ascorbic acid (1). A previous turnover rate study showed that this increase results from an acceleration in the rate of synthesis of L-ascorbic acid (2), but there is no understanding of the mechanism by which drugs exert this effect. Tracer studies in rats, indicating that the lactone of D-glucuronic acid is an intermediate in the biosynthesis of L-ascorbic acid from D-glucose, suggest that the initial effect of the drugs may be on the synthesis of D-glucuronic acid (3, 4). Since many drugs are known to be detoxified in the body by being excreted in urine as conjugates with D-glucuronic acid, the possibility has been pointed out previously that the increased excretion of L-ascorbic acid in response to drugs may also represent a detoxification mechanism (1).

One of the drugs which produces a marked increase in the excretion of L-ascorbic acid is barbitol (1). Unlike most drugs, it is not metabolized *in vivo*, but after administration to man and dog it can be recovered unchanged in urine (5-7). This observation is of considerable interest since it offers the possibility of separating the effect of drugs on L-ascorbic acid and D-glucuronic acid synthesis from their detoxification in the body either by metabolic transformation or by conjugation. For this reason the present investigation was carried out to determine the effect of barbitol on L-ascorbic acid and D-glucuronic acid excretion in rats, dogs, and guinea pigs. In addition, the incorporation of D-glucose-1-C¹⁴ into urinary D-glucuronic acid and L-ascorbic acid was compared in normal and barbitol-treated rats. The results show that barbitol, despite the fact that it is not metabolized, produces in rats and dogs a marked increase in the urinary excretion of D-glucuronic acid and L-ascorbic acid. In guinea pigs, only an increase in excretion of D-glucuronic acid is observed since this species is unable to synthesize L-ascorbic acid.

EXPERIMENTAL

Radioactive Material and Procedures—D-Glucose-1-C¹⁴ with a specific activity of 10 μ c per mg was obtained from the National Bureau of Standards, Washington, D C The method for collection and preparation of samples and their assay for C¹⁴ were the same as those used previously (8) The barium salt of the *p*-bromophenylosazone of D-glucuronic acid was counted as a disk on filter paper, the appropriate correction being made for self-absorption

Experimental Animals—Male albino rats of the Wistar strain were maintained on a basal diet of evaporated milk and water for at least 7 days before each experiment The diet of the hypophysectomized¹ and adrenalectomized rats was supplemented with saline Male guinea pigs were kept on Rockland Farm guinea pig diet and water during the experiments Female mongrel dogs were employed and were given a stock diet (Big Red Kibbled dog cakes²) and water

Analytical Methods—L-Ascorbic acid was determined by titration with indophenol dye (9), and D-glucuronic acid was estimated by the naphthoresorcinol method (10)

Barbital was determined in urine of rats and guinea pigs by a modification of a spectrophotometric method previously described (11) In brief, the method consisted of adjusting the urine to pH 5.0, followed by its extraction with ethyl ether The organic phase was shaken with Norit, which served to remove the major fraction of urine blank material but negligible amounts of barbital The drug was then extracted from ethyl ether into 0.5 N NaOH, in which solution its absorption at 255 m μ was measured in the Beckman spectrophotometer The technique of comparative distribution ratios permitted the assay of the specificity of the method for barbital in urine (12) The partition ratios of the substance extracted from urine were identical with those of authentic barbital in a series of two-phase systems consisting of ethyl ether and various aqueous buffers Similar values for barbital in urine were obtained by modification of a procedure used for determination of pentobarbital, except that ethylene dichloride was used as the solvent and the drug was measured in the ultraviolet region at 255 m μ in 0.5 N NaOH (13)

Isolation Procedures—The 24 hour urine samples, preserved either with 0.5 ml of toluene or 5 ml of 10 per cent oxalic acid, were diluted to 100 ml with water L-Ascorbic acid was isolated after addition of 300 mg carrier to a 30 ml aliquot of urine by a specific ion exchange procedure used previously (8) D-Glucuronic acid was isolated as the lactone as

¹ Hypophysectomized rats were purchased from the Hormone Assay Laboratories, Inc., Chicago, Illinois

² G. L. F. Exchange Company, Canandaigua, New York

follows 300 mg of carrier D-glucuronic acid were added to a 30 ml aliquot of the urine sample and the solution was passed through an Amberlite IR-4B anion exchange column in the acetate form (8). The adsorbed D-glucuronic acid was eluted with 2 N formic acid and the eluate was evaporated rapidly to dryness *in vacuo* at 50°. The D-glucuronic acid was converted to its lactone by crystallization from glacial acetic acid. The resulting D-glucuronolactone was dissolved in H₂O and passed again through the Amberlite IR-4B column. The effluent containing the D-glucuronolactone was evaporated rapidly to dryness *in vacuo* at 50°. D-Glucuronolactone was recrystallized three times from glacial acetic acid. Radioactive purity was established when upon recrystallization no change was found in specific activity. In addition, two derivatives, the barium salt of the *p*-bromophenylosazone and potassium acid saccharate (14), prepared from the same sample of D-glucuronolactone, had identical molar specific activities.

Results

Fate of Barbitol in Rats and Guinea Pigs—The excretion of barbitol was measured after its intraperitoneal administration to two rats and two guinea pigs in doses of 46 to 50 mg, respectively (Table I). It will be noted that essentially all of the administered drug is recovered in the urine during a 3 to 4 day period, an average of 98 per cent for both species. These results are in agreement with those obtained in man and dog, which show that barbitol is not metabolized (5-7).

Effect of Barbitol on L-Ascorbic Acid and D-Glucuronic Acid Excretion—The effect of different dosages of barbitol on the urinary excretion of L-ascorbic acid and D-glucuronic acid was measured (Table II). It will be noted that barbitol produces a significant effect on L-ascorbic acid and D-glucuronic acid excretion in doses of as little as 10 mg daily.

The effect of barbitol in intraperitoneal doses of 150 mg daily on urinary excretion of D-glucuronic acid was measured in four guinea pigs (Table III). Like the rat, a significant increase in D-glucuronic acid was observed. The guinea pig, however, differs from the rat in being unable to convert D-glucuronolactone to L-ascorbic acid (4).

The effect of barbitol on the daily urinary excretion of L-ascorbic acid and D-glucuronic acid was measured in two dogs after they received daily 50 mg per kilo of barbitol by stomach tube for 5 days. The average daily excretion of L-ascorbic acid and D-glucuronic acid before administration of the drug was 47 and 373 mg, respectively. In the case of one dog, the daily excretion of L-ascorbic acid and D-glucuronic acid increased to 218 and 717 mg, respectively, after drug administration. For the second dog the daily excretion of L-ascorbic acid and D-glucuronic acid increased

to 161 and 725 mg, respectively. In addition, similar studies were carried out in two other dogs which received daily 100 mg per kilo of Chlore-tone by stomach tube for 5 days. The daily urinary excretion of L-ascorbic

TABLE I
*Urinary Excretion of Barbitol by Rats and Guinea Pigs**

Collection period	Amount of barbitol in urine	
	Rat A	Rat B
<i>days</i>	<i>mg</i>	<i>mg</i>
0-1	27 1	35 0
1-2	11 3	7 7
2-3	3 5	2 3
3-4	0 7	0 4
	Guinea pig C	Guinea pig D
1-2	39 0	42 0
2-3	15 0	4 3

* The rats and guinea pigs received 46 and 50 mg, respectively, of sodium barbitol by intraperitoneal injection. All the values are expressed in terms of sodium salt.

TABLE II
*Daily Urinary Excretion of D-Glucuronic and L-Ascorbic Acids after Administration of Barbitol to Rats**

Dose of barbitol†	No. of rats	D-Glucuronic acid	L-Ascorbic acid
<i>mg per day</i>		<i>mg per day</i>	<i>mg per day</i>
0 0	30	16 ± 2 6‡	0 82 ± 0 50‡
10	6	28 ± 2 0	6 3 ± 0 90
50	6	37 ± 4 8	17 ± 3 4
150	4	63 ± 5 5	23 ± 3 2

* The average value for urinary excretion of each compound after the rats had received the indicated dosage for 4 days. Urine samples were preserved by collection in 5 ml of 10 per cent oxalic acid.

† Barbitol was given as sodium salt dissolved in an evaporated milk diet. The rats weighed from 275 to 325 gm.

‡ Average deviation.

acid increased from an average control value of 68 mg before administration of the drug to a maximal value for one dog of 287 mg and for the other, 576 mg.

Effect of Glucuronide-Producing Drugs on L-Ascorbic Acid Excretion—

Additional studies were carried out in rats to determine whether compounds such as borneol, α -naphthol, and phenolphthalein, which are excreted chiefly as glucuronides (15), increase the excretion of L-ascorbic acid. These drugs have essentially no effect on L-ascorbic acid excretion,

TABLE III

*Urinary Excretion of D-Glucuronic Acid after Administration of Barbitol to Guinea Pigs**

Day No	Dosage†	Urinary D glucuronic acid			
		Guinea pig 1	Guinea pig 2	Guinea pig 3	Guinea pig 4
	<i>mg per day</i>	<i>mg per day</i>	<i>mg per day</i>	<i>mg per day</i>	<i>mg per day</i>
Control	0 0	33	36	28	28
"	0 0	29	30	37	33
"	0 0	30	25	40	37
1	150	48	69	38	58
2	150	84	72	60	67
3	150	82		55	

* Urine samples were preserved by collection in toluene

† Administered as sodium barbitol divided into two doses by intraperitoneal injection. The guinea pigs weighed from 425 to 450 gm

TABLE IV

*Effect of Glucuronide-Producing Drugs on L-Ascorbic Acid Excretion by Rats**

Drug	Dosage	No of rats	L Ascorbic acid	D Glucuronic acid
	<i>mg per day</i>		<i>mg per day</i>	<i>mg per day</i>
None		30	0 82 \pm 0 50†	16 \pm 2 6†
Borneol	100	3	0 94 \pm 0 25	76 \pm 13
α -Naphthol	100	3	0 53 \pm 0 07	56 \pm 4 0
Phenolphthalein	100	4	0 60 \pm 0 16	31 \pm 4 0

* The average value for urinary excretion after 2 days on each dosage schedule. The drug was administered by stomach tube as a homogenate in evaporated milk

† Average deviation

but increase markedly the excretion of D-glucuronic acid (Table IV). These results are in marked contrast to those obtained with barbitol, which increases the excretion of both L-ascorbic and D-glucuronic acids.

The effect of borneol on L-ascorbic acid excretion by rats receiving either barbitol or Chloretone was also measured (Table V). The results show that borneol does not affect the excretion of L-ascorbic acid by rats receiving these drugs.

TABLE V

*Effect of Borneol on L-Ascorbic Acid Excretion by Rats Receiving Barbital and Chloretone**

Experiment No	Drug	Dosage*	Urinary ascorbic acid
		mg per day	mg per day
1	Barbital	50	20 \pm 1 0†
	" + borneol	50 + 100	20 \pm 2 0
2	Chloretone	50	35 \pm 6 0
	" + borneol	50 + 100	30 \pm 5 0

* The rats received either barbital or Chloretone for 7 days, and on the last 2 days the animals also received borneol. The drugs were administered by stomach tube. Three rats were used in each experiment.

† Average deviation

TABLE VI

Incorporation of Glucose-1-C¹⁴ into Urinary D-Glucuronic Acid and L-Ascorbic Acid in Normal and Drug-Treated Rats

Experiment No	Drug*	Per cent of dose in	
		D Glucuronic acid	L Ascorbic acid
R-309†	None	<0 04	
R-344B†	"	<0 02	
R-1†	"		0 02
R-12†	"		0 01
R-303	Barbital	0 45	0 15
R-306	"	0 45	0 22
R-327†	"	0 34	
R-346A†	Chloretone	0 11	
R-346C	"	0 18	
R-3†	"		0 55
R-4†	"		0 45

* 150 mg of barbital were administered daily by intraperitoneal injection, divided into two equal doses. 50 mg of Chloretone were administered by stomach tube as a homogenate in evaporated milk. The animal received the drug at least 4 days before the experiment. The animals weighed from 275 to 325 gm. Weighed quantities of D-glucose-1-C¹⁴ (10 to 20 mg) were administered to each animal by intraperitoneal injection.

† In these experiments urine was preserved with toluene. For the remaining experiments, 5 ml of 10 per cent oxalic acid were used.

‡ Previously published experiments (16)

Radioactive Experiments—The incorporation of D-glucose-1-C¹⁴ into D-glucuronic acid and L-ascorbic acid was compared in normal rats and in those receiving barbital and Chloretone (Table VI). It will be noted

that administration of barbital produces a marked increase in the conversion of D-glucose to D-glucuronic acid from an average of <0.03 per cent in normal rats to an average of 0.40 per cent in rats receiving the drug. Barbital also produced about a 10-fold increase in the incorporation of D-glucose-1-C¹⁴ into L-ascorbic acid.

The conversion of labeled D-glucose to D-glucuronic acid and L-ascorbic acid in rats receiving Chloretone averaged 0.15 per cent and 0.50 per cent,

TABLE VII

Effect of Barbital and Chloretone on L-Ascorbic Acid Excretion in Normal, Hypophysectomized, and Adrenalectomized Rats

Drug	Dosage schedule*	Normal		Hypophysectomized		Adrenalectomized	
		No of rats	L Ascorbic acid in urine	No of rats	L Ascorbic acid in urine	No of rats	L Ascorbic acid in urine
			<i>mg per day</i>		<i>mg per day</i>		<i>mg per day</i>
None		8	0.28 ± 0.04†	22	0.26 ± 0.12†	8	0.35 ± 0.15†
Barbital	12.5 mg for 2 days, 25 mg for 1 day by intraperitoneal injection	8	3.2 ± 0.91	6	0.37 ± 0.45		
Barbital	25 mg for 2 days by stomach tube	5	4.9 ± 0.81	3	0.19 ± 0.02	3	3.2 ± 0.46
Chloretone	12.5 mg for 3 days by stomach tube	8	10.8 ± 1.63	5	2.4 ± 1.73	5	12.1 ± 1.7
Chloretone	25 mg for 1 day by stomach tube	7	7.2 ± 1.95	3	0.24 ± 0.02		

* Excretion of L-ascorbic acid was measured during the last day of drug administration. The rats weighed from 190 to 210 gm.

† Average deviation.

respectively. Previous studies have shown that Chloretone has an effect similar to barbital in stimulating the excretion of L-ascorbic and D-glucuronic acids (17). Since little information is available on the fate of Chloretone, it was not known whether this increase in D-glucuronic acid excretion represents free D-glucuronic acid or glucuronides. The method used in measuring D-glucuronic acid does not distinguish between the free and the conjugated form (10). The results of the present tracer study show that at least a part of the D-glucuronic acid excreted in urine in response to Chloretone is present in the free form.

Experiments with Hypophysectomized and Adrenalectomized Rats—The

effect of different dosages of barbital and Chloretone upon excretion of L-ascorbic acid by normal, hypophysectomized, and adrenalectomized rats was compared (Table VII). It will be noted that administration of either barbital or Chloretone has little or no effect on L-ascorbic acid excretion by hypophysectomized rats. However, administration of these drugs to adrenalectomized rats produces an increase in L-ascorbic acid excretion similar to that observed in normal rats.

DISCUSSION

The results presented in this study show that barbital produces a marked increase in urinary excretion of both L-ascorbic acid and D-glucuronic acid in rats and dogs and of D-glucuronic acid only in guinea pigs. The observation that barbital is neither metabolized nor conjugated in the body indicates that its effect on L-ascorbic acid and D-glucuronic acid synthesis occurs independently of any known detoxication mechanism.

The results of the experiments in which the incorporation of D-glucose-1-C¹⁴ into urinary L-ascorbic acid and D-glucuronic acid was measured in normal rats and in those receiving either barbital or Chloretone further point out the striking effect of the drug on the synthesis of these compounds. Of particular importance, the isotopic dilution method employed in these experiments furnishes evidence for the presence of free D-glucuronic acid in urine of rats receiving barbital and Chloretone. Data for the formation of free D-glucuronic acid in animals have been meager (18, 19). Evidence has been presented previously by Smith and Williams (20, 21) for free D-glucuronic acid in urine of rabbits receiving aniline and phenetidine, but it was thought to originate from the breakdown of labile glucuronides of the administered compound.

Administration of barbital or Chloretone to rats apparently stimulates the synthesis of L-ascorbic acid via the following pathway: D-glucose \rightarrow D-glucuronolactone \rightarrow L-gulonolactone \rightarrow L-ascorbic acid. Evidence for this scheme has come from experiments in both normal and Chloretone-treated rats in which the incorporation of C¹⁴ in L-ascorbic acid was measured after administration of various tracers, including carbon 1, carbon 6, and uniformly labeled D-glucose (22-24, 16), carboxyl, and uniformly labeled D-glucuronolactone (3, 4), and carboxyl-labeled L-gulonolactone³ (4). Further evidence for this pathway is found in the present study in that barbital and Chloretone stimulate the conversion of D-glucose-1-C¹⁴ to D-glucuronic acid. Other studies have shown that administration of these drugs to rats also increases the conversion of D-glucose-1-C¹⁴ to uri-

³ The possibility that D-glucuronic acid and L-gulonic acid as well as their lactones are intermediates in the biosynthesis of L-ascorbic acid has been pointed out recently from *in vitro* experiments (25).

nary L-gulonic acid (26) The amount of D-glucuronic acid and L-ascorbic acid excreted in urine represents only a small fraction of the total D-glucose metabolized via this pathway For instance, administered D-glucuronolactone and L-gulonolactone are extensively oxidized to CO_2 and only a small fraction excreted as their respective acids in urine (3, 4) In addition, only a part of L-ascorbic acid synthesized each day in rats treated with drugs is excreted in urine, and the remainder is either oxidized to CO_2 or excreted in urine as various metabolites (2)

Barbital is one of the many drugs which increases the urinary excretion of L-ascorbic acid These include various barbiturates, other hypnotic drugs such as Chloretone, paraldehyde, and Trional (sulfonethylmethane), and certain antipyretic and analgesic drugs such as antipyrine and aminopyrine (1) Since there is an apparent lack of structural specificity for this effect on L-ascorbic acid excretion, the possibility must be considered that the drugs act in some indirect way on carbohydrate metabolism, resulting in D-glucose being metabolized via D-glucuronolactone Possible hormonal control over this phenomenon is suggested from the results, which show that the effect of Chloretone and barbital on L-ascorbic acid excretion is considerably less in hypophysectomized rats than in normal rats The adrenals are apparently not involved, however, since administration of these drugs to adrenalectomized rats produces about the same increase in L-ascorbic acid excretion as that observed in normal rats

SUMMARY

Barbital produces a marked increase in urinary excretion of L-ascorbic acid and D-glucuronic acid in rats and dogs In the guinea pig, a species which is unable to synthesize L-ascorbic acid, an increase in D-glucuronic acid excretion only occurs The incorporation of D-glucose-1- C^{14} into free D-glucuronic acid in urine is markedly enhanced upon administration of barbital and Chloretone to rats

The effect of barbital on L-ascorbic acid and D-glucuronic acid excretion is apparently not related to any known detoxification mechanism since barbital is neither metabolized nor conjugated but is excreted unchanged in urine The results point out a possible additional mechanism for D-glucuronic acid formation in response to drugs other than that via glucuronide synthesis

Barbital and Chloretone have little or no effect on L-ascorbic acid excretion in hypophysectomized rats, suggesting possible hormonal control over L-ascorbic acid synthesis in response to drugs

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EFFECTS OF AMETHOPTERIN ON NUCLEIC ACID METABOLISM IN MITOTIC AND NON-MITOTIC GROWTH*

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Of the protein and ribose nucleic acid (RNA) normally present in liver, approximately one-half is lost when adult male rats are fasted for 6 days (1). Neither during this fasting period nor during the recovery period after the animals are returned to a normal diet is there a significant change in the total number of cells or in the total content of deoxyribose nucleic acid (DNA) in the liver (1-3). Substantially no mitosis or endomitosis takes place. Thus, the rapid synthesis of RNA and protein which accompanies refeeding is associated with cell enlargement, *i.e.* non-mitotic growth.

In contrast, regeneration of liver tissue after partial hepatectomy is accomplished by mitotic reproduction of the cells present after the operation (4). The visible onset of mitosis takes place between 24 and 36 hours after the operation, but Price and Laird (5) have shown that a rapid synthesis of DNA and other nuclear and cytoplasmic components begins approximately 12 hours after the operation. It is becoming increasingly evident that, before the visible onset of mitosis, there is a period in which many cell components are synthesized in preparation for cell division (5-8).

The present study is concerned with the effects of amethopterin (4-amino-*N*¹⁰-methylpteroylglutamic acid), a known antagonist of folic acid and citrovorum factor (9-11), on the synthesis of nucleic acid and protein which accompanies both the mitotic growth of rat liver upon partial hepatectomy and the non-mitotic growth of the same tissue during refeeding after fasting.

Materials and Methods

Male albino rats¹ weighing 200 to 250 gm were used. The food consisted of Friskies dog pellets, and the animals were maintained on this diet for at least 1 week before being used in experiments.

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¹ Obtained from the Holtzman Rat Company, Madison, Wisconsin.

In the experiments on recovery from fasting (Experiments I to IV, Tables I and II), the animals were fasted for 6 days and then allowed free access to food. Throughout all of the experiments, the rats had access to water. The treated animals received amethopterin² (2 mg per kilo) in a single intraperitoneal injection each day, the first injection being administered 1 hour before the fasted animals resumed feeding. The control animals were pair-fed to those receiving amethopterin.

Three studies of regenerating liver were made. In the first two (Experiments V and VI, Table III), the animals received amethopterin (2 mg per kilo) in a single intraperitoneal injection each day for 4 days (Experiment V) or 5 days (Experiment VI) and were then subjected to partial hepatectomy by the procedure of Higgins and Anderson (12). Beginning 3 hours after the operation, each animal received amethopterin by subcutaneous injection every 6 hours (0.8 mg per kilo), and they were killed 30 hours after the operation. In the third of the regenerating liver experiments (Experiment VII), the animals received no amethopterin before partial hepatectomy; treatment after the operation was the same as that in the other regenerating liver experiments, except that the amethopterin dose was 2 mg per kilo for each injection. In each case, the control animals were pair-fed to those receiving amethopterin.

The procedure for the homogenization and fractionation of the liver tissue has been described previously (13). Samples of the tissue homogenates and cell fractions were analyzed for protein nitrogen by a nesslerization procedure (14) and for nucleic acids by the method of Schneider (15). Nuclei were counted in the unfractionated homogenates as described previously (16).

3 hours before being killed, each animal received glycine-2-C¹⁴ (4 mg per kilo, 67 μ c per kilo)³ and inorganic phosphate-P³² (20 μ c per kilo)³ in a single intraperitoneal injection, except that in Experiment I the P³² dosage was 200 μ c per kilo. The procedure for the isolation of protein and nucleic acid samples for determination of specific activity was based on the method described by Barnum and Huseby (17) and was set up in this laboratory by Dr. Oddvar Nygaard (1). The P³² radioactivity of the nucleic acid extracts was measured in an annulus counter⁴ which registered only P³², since its walls were thick enough to absorb substantially all of the

² The amethopterin was generously donated by Dr. J. M. Rueggsegger, of the Lederle Laboratories Division of the Union Carbide and Carbon Company.

³ The glycine-2-C¹⁴ was obtained from Tracerlab, Inc., on allocation from the United States Atomic Energy Commission. The orthophosphate-P³² was supplied by Dr. E. C. Albright of the Department of Medicine, University of Wisconsin, on allocation from the United States Atomic Energy Commission.

⁴ Obtained from the Radiation Counter Laboratories, Inc., Skokie, Illinois.

C^{14} radioactivity For measurement of the C^{14} radioactivity of these solutions, aliquots were subjected to wet combustion (Van Slyke-Folch), the $C^{14}O_2$ thus separated from the P^{32} was trapped in NaOH and converted to $BaCO_3$ for counting.⁵ The protein samples were counted under an end window counter, with and without an aluminum disk, which absorbed substantially all of the C^{14} radioactivity and reduced the P^{32} count to 73 per cent of that obtained without the absorber, the data so obtained were used to calculate the C^{14} radioactivity of the protein samples

The specific activity of the uncombined glycine was determined by the procedure described previously (18), except that the dimedon precipitate was dissolved in dilute alkali and reprecipitated by the addition of 2 to 3 drops of glacial acetic acid. The specific activity of the acid-soluble phosphorus was determined by measuring the P^{32} activity and total phosphorus content of a trichloroacetic acid extract prepared from each homogenate

Results

Experiments on Recovery from Fasting—During the 1st day of refeeding, the food intake of the animals receiving amethopterin was the same as that of control animals allowed to eat *ad libitum*, thereafter the food intake decreased rapidly, reaching zero by the 4th day of refeeding, at which time the animals were moribund. In these rats, the wall of the intestine was very thick and the lumen was filled with mucus. The quantities of RNA and protein nitrogen in the cell fractions of the animals during refeeding after fasting are shown in Table I. Since it had been found that there was no change in the number of cells in the liver during fasting and refeeding (1-3), the values may be expressed either in terms of the average liver or of the average liver cell, in Table I they are given in terms of mg per average liver. After 2 days of refeeding, the values for the animals treated with amethopterin fell between those for 1 and 2 days of refeeding in control animals fed *ad libitum*, and were slightly higher than those for pair-fed controls. After 4 days of treatment with amethopterin, the quantities of RNA and protein nitrogen in the average liver were lower than after 2 days of treatment, as would be expected from the reduced food intake, but here again they were higher than the corresponding values in pair-fed controls.

The data in Table II represent the relative specific activity of the nucleic acid and protein in these animals during refeeding after fasting, they are expressed as (counts per minute per mg divided by counts per minute

⁵ We are grateful to Mrs Edith Wallestad and Mrs Dorothy McManus for assistance in the measurement of C^{14} radioactivity, under the general supervision of Dr C Heidelberg

per microgram of uncombined glycine or acid-soluble P) ⁶ It is evident that treatment with amethopterin for 2 days produced no significant effect on the incorporation of either label into the RNA in the liver cell fractions After 4 days of treatment, the labeling of these fractions tended to be higher than in the pair-fed controls Under the conditions used,

TABLE I

Influence of Amethopterin Treatment on Restoration of Ribose Nucleic Acid and Protein in Rat Liver during Refeeding after Fasting

Cell fraction	Fasted 6 days	Refeeding after fasting							
		1 day	2 days				4 days		
		Group A*	Group A	Group B	Group C	Group B	Group C		
Ribose nucleic acid, mg per liver									
Homogenate	41 0	62 7	76 7	69 7	74 8	71 5	61 2	71 0	41 0
Nuclei	10 8	14 0	10 8	17 8	11 6	16 2	11 9	4 7	10 0
Mitochondria I	1 1	1 3	1 6	1 4	2 4	2 1	1 9	2 0	0 8
“ II	5 8	16 5	28 6	13 9	11 9	10 7	5 3	9 5	2 8
Microsomes	15 6	19 6	28 5	17 1	16 7	26 0	13 4	17 3	10 0
Supernatant	8 9	13 0	15 7	14 0	13 6	15 2	11 2	11 8	10 2
Protein nitrogen, mg per liver									
Homogenate	142†	187	214	200	204	179	194	170	124
Nuclei	44 5	77 4	37 8	53 3	47 4	47 0	46 5	34 5	42 9
Mitochondria I	22 0	13 5	23 5	22 2	30 4	32 8	30 8	30 9	20 0
“ II	13 5	32 0	41 1	33 0	33 0	18 8	16 7	23 6	5 7
Microsomes	17 9	24 8	35 1	27 0	30 0	30 3	28 2	30 8	17 1
Supernatant	43 3	53 4	74 8	60 5	70 0	84 8	61 9	57 5	38 8

* Group A, animals fed *ad libitum*, Group B, animals which received amethopterin (2 mg per kilo) in a single intraperitoneal injection each day and were fed *ad libitum*, Group C, controls, pair-fed to animals receiving amethopterin The two mitochondria fractions are described in the text

† In each case, measurements were made on pooled material from four animals

the incorporation of both labels into the DNA was too low to permit valid conclusions regarding the effects of amethopterin treatment on the label-

⁶ Basing the relative specific activities of nucleic acids and proteins on the specific activity of uncombined glycine or acid-soluble phosphate at the time of sacrifice is obviously arbitrary, since we do not know the specific activity of the immediate precursors, particularly in the case of the nucleic acids However, the usefulness of the method is indicated by the good agreement in the P³² relative specific activity of liver RNA in Experiments I and II (Table II) despite a 10 fold difference in the level of P³² dosage

TABLE II

Relative Specific Activities of Ribose Nucleic Acid and Protein in Tissues of Rats Treated with Amethopterin during Refeeding after Fasting*

	2 days refeeding				4 days refeeding			
	Amethopterin		Control		Amethopterin		Control	
	Experiment No		Experiment No		Experiment No		Experiment No	
	I	II	I	II	III	IV	III	IV
Liver RNA (C ¹⁴)	(50)†	(51)	(48)	(50)	(73)		(65)	
Nuclei	9 5	10 0	9 7	6 4	17 9		16 6	
Mitochondria II	1 5	0 4	0 4	0 4	6 1		0 7	
Microsomes		0 5		0 3	1 5		0 8	
Supernatant	2 0	1 7	6 1	0 6	3 5		1 8	
Liver RNA (P ³²)	(184)	(40)	(178)	(41)	(49)		(31)	
Nuclei	130	136	96	127	110		100	
Mitochondria II	12 4	9 8	7 2	5 2	16 0		18 3	
Microsomes	25 2	13 8	12 4	5 7	14 4		16 2	
Supernatant	38 7	36 7	25 3	24 2	36 2		45 1	
Liver protein (C ¹⁴)	(50)	(51)	(48)	(50)				
Nuclei	14	15	14	13				
Mitochondria I	7 9	8 9	8 2	7 7				
“ II	11	14	13	12				
Microsomes	11	20	19	17				
Supernatant	16	13	13	11				
Pancreas RNA (C ¹⁴)						0 5 (96)		0 7 (67)
“ “ (P ³²)						1 1 (11)		0 5 (9)
Small intestine RNA (C ¹⁴)					16 (69)	22 (57)	65 (29)	34 (38)
Small intestine RNA (P ³²)					58 (19)	3 0 (18)	44 (17)	10 (13)
Small intestine DNA (C ¹⁴)						1 5 (57)		18 (38)
Small intestine DNA (P ³²)						0 4 (18)		8 (13)
Spleen RNA (C ¹⁴)						1 2 (54)		1 3 (39)
“ “ (P ³²)						4 2 (8 1)		12 (9)
“ DNA (C ¹⁴)						1 0 (54)		13 (39)
“ “ (P ³²)						0 (8 1)		13 (9)

* Relative specific activities are expressed as (counts per minute per mg. of RNA, or protein divided by counts per minute per microgram of glycine, or acid-soluble P)

† The numbers in parentheses give the observed specific activity of the uncombined glycine, or acid-soluble phosphate, expressed as counts per minute per microgram. In each case, measurements were made on pooled material from four animals

ing of liver DNA in these animals. In the pancreas, as in the liver, incorporation of both labels into DNA was extremely low and amethopterin

TABLE III

Influence of Amethopterin Treatment on Quantities and Specific Activities of Cell Constituents in Livers of Rats Regenerating after Partial Hepatectomy

Experiment No	DNA			Glycine	Acid soluble P	RNA			Protein nitrogen		RNA DNA	RNA Protein N	
	μ gm per gm	C ¹⁴	P ³²			μ gm per gm	C ¹⁴	P ³²	μ gm per gm	C ¹⁴			
V Amethopterin	(1)*	12.2	3.83†	3.83†	49.2†	13.7†	33.5	3.23†	5.21†	114	20.1†	2.75	0.294
	(2)	12.4	3.52	0.99	46.2	15.6	33.9	2.74	2.27	109	23.2	2.74	0.311
	(3)	13.3	2.68	1.06	48.3	37.4	43.7	2.59	1.20	151	19.0	3.28	0.289
	(4)	15.6	7.50	6.07	43.2	13.9	41.2	1.78	2.70	136	24.4	2.64	0.303
Mean		13.4	4.38	3.00			38.1	2.58	2.84	128	21.7	2.85	0.299
Control	(1)	15.9	31.2	10.4	26.9	16.1	50.3	22.3	8.45	137	45.1	3.16	0.317
	(2)	16.4	39.8	11.5	26.8	12.8	39.6	17.8	9.84	128	40.8	2.42	0.309
	(3)	18.4	35.5	18.0	29.8	12.0	56.7	13.8	9.06	169	39.4	3.06	0.335
	(4)	15.6	13.2	7.2	33.4	14.1	41.7	12.3	8.72	125	34.8	2.68	0.333
Mean		16.6	29.9	11.8			47.1	16.5	9.02	140	40.0	2.83	0.323
VI Amethopterin	(1)	11.1	6.78	2.69	52.0	20.3	48.5	14.7	4.23	120		4.37	0.404
	(2)	10.8		0.47	37.4	21.3	48.5	24.2	4.65	122		4.49	0.398
	(3)	9.5	11.0	4.95	51.8	22.4	48.3	14.1	4.35	134		5.08	0.361
Mean		10.5	8.9	2.70			48.4	17.7	4.41	125		4.65	0.388
Control	(1)	19.0	26.3	5.79	43.8	28.8	85.0	76.8	8.32	205		4.47	0.415
	(2)	16.2	29.5	4.32	44.6	30.4	64.3	94.2	8.75	171		3.97	0.376
	(3)	18.5		6.10	76.7	21.5	72.2	32.6	9.02	175		3.90	0.413
Mean		17.9	27.9	5.40			73.8	67.9	8.70	184		4.12	0.401
VII Amethopterin		14.3	2.46	8.1	89.5	8.8	59.7	1.84	8.3	168		4.18	0.356
Control		14.0	5.26	8.1	84.1	15.0	61.8	6.46	8.5	144		4.31	0.428

* In Experiments V and VI, each measurement was made on material from one animal, in Experiment VII, each measurement was made on pooled material from four animals

† The relative specific activities of nucleic acids and protein are expressed as (counts per minute per mg of nucleic acid, or protein divided by counts per minute per microgram of uncombined glycine, or acid-soluble P)

‡ The specific activity of uncombined glycine, or acid-soluble P, is expressed as counts per minute per microgram

produced no significant effect on the labeling of RNA. In the small intestine and spleen, on the other hand, there was extensive incorporation of both labels into the DNA, and both of these processes were inhibited drastically by the amethopterin treatment. There was also some indication that the amethopterin treatment reduced the incorporation of the two labels into the RNA in these tissues.

Experiments on Regenerating Liver—Data on the quantities and labeling of nucleic acid and protein in the livers of animals after partial hepatectomy are given in Table III, the quantities are expressed as $\text{gm} \times 10^{-12}$ of nucleic acid or protein nitrogen per cell (calculated on the basis of one nucleus per cell), and the specific activities are expressed as in Table II. In Experiments V and VI, the amethopterin not only inhibited the incorporation of both labels into DNA and RNA, but prevented the increase in the quantities of these substances per cell which normally occur during the first 24 to 30 hours after partial hepatectomy (5). In these experiments, there is considerable variation in the extent of the increase in DNA per cell in the controls and in the degree of inhibition of the DNA increase by the amethopterin treatment, and yet, in comparison with its effect on the increase of DNA, this treatment produced very little change in the ratio of RNA to DNA or in the ratio of RNA to protein nitrogen. Thus, when the amethopterin produced an inhibition of the increase of DNA, it produced a proportional inhibition of the increase in RNA and protein nitrogen. The increases of RNA and particularly of protein per cell in these experiments are somewhat larger than those reported for normal rats by Price and Laird (5), but in the present experiments the period of regeneration was 30 rather than 24 hours, and the initial quantities of RNA and protein per cell were lower than normal, due to the reduction in food intake produced by the pretreatment with amethopterin.

In Experiment VII, in which the rats were not pretreated with amethopterin before partial hepatectomy but received frequent large doses thereafter, the amethopterin treatment inhibited the incorporation of glycine-2- C^{14} into both DNA and RNA, but this did not interfere with the normal increase in the amounts of these substances per cell, or with their labeling by P^{32} .

DISCUSSION

It is generally agreed that the action of the antifolic drugs is directed chiefly against tissues in which there is active cell division, and this was true in the present experiments. The labeling of the nucleic acids was inhibited only in the spleen and small intestine, but not in the liver and pancreas of the animals recovering from fasting. When the non-mitotic metabolism of the liver was diverted to preparation for cell division, upon

partial hepatectomy, the nucleic acid metabolism in this organ became susceptible to inhibition by amethopterin (*cf* Nanetti (19)). In this case, the drug produced an inhibition of the net synthesis of RNA and protein that was proportional to the inhibition of the net synthesis of DNA, suggesting that these syntheses are parts of an integrated process in which the cells prepare for division and that the amethopterin inhibited the onset of this process.

Apparently the synthesis of RNA is not affected directly by amethopterin, but only indirectly as a result of an inhibition of the preparation for cell division, and consequently only the RNA metabolism associated with mitosis is affected. Furthermore, the inhibitory effect of this drug *in vivo* is probably not on a phase of nucleic acid synthesis common to RNA and DNA, as would be the case in the incorporation of 1-carbon units into the purines.

It is evident that inhibition of the incorporation of 1-carbon units into the purines and thymine is not immediately sufficient to prevent the synthesis of DNA, because the normal net synthesis of RNA and DNA, and their labeling by P^{32} , was not inhibited when the administration of amethopterin at a high dose level was confined to a brief period after partial hepatectomy, even though this resulted in a marked inhibition of the incorporation of glycine into both nucleic acids (20-22).

Brief administration of amethopterin even at a high dose level was ineffective, and prolonged administration was required in order to produce effective inhibition of nucleic acid synthesis. Consequently, it appears that the inhibitory effect is produced by the depletion of some critical material (23, 24), the product of a reaction that is susceptible to inhibition by amethopterin. This product might well be a thymine derivative (25-27), or it might be a cofactor for which amethopterin is not a competitive antagonist. It is not improbable that some early stage in the synthesis of DNA, which is susceptible to amethopterin, is responsible for triggering the integrated synthesis of cell constituents in preparation for mitosis.

SUMMARY

The present study deals with the effects of amethopterin on the changes that take place in the net quantities of deoxyribose nucleic acid, ribose nucleic acid, and protein and in the incorporation of glycine-2- C^{14} and inorganic phosphate- P^{32} into the cell constituents during the mitotic growth of rat liver following partial hepatectomy and during the non-mitotic growth of this tissue that accompanies refeeding after fasting.

Nucleic acid synthesis was inhibited only when it was associated with the preparation for cell division. This inhibition seemed to be produced

by depletion of some critical material, because prolonged administration of the drug was required to produce it. Apparently ribose nucleic acid synthesis is not inhibited directly *in vivo*, but only indirectly as a result of the inhibition of the preparation for mitosis.

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STUDIES ON THE MECHANISM OF FORMALDEHYDE INCORPORATION INTO SERINE*

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Previous work has demonstrated that synthetic 5,6,7,8-tetrahydrofolic acid¹ stimulates the incorporation of formaldehyde into serine (2-4). In the present investigation it is shown that formaldehyde reacts non-enzymatically with THFA to form precursors of the serine β -carbon.

Materials and Methods

Preparation and Assay of Reduced Pteridines—The THFA used in these experiments was prepared by hydrogenating 1 gm of commercial folic acid (Nutritional Biochemicals Corporation, Cleveland, Ohio) in 100 ml of glacial acetic acid with 500 mg of platinum oxide catalyst (J H Bishop Company, Malvern, Pennsylvania) for 5 hours at 25° (5). The uptake of hydrogen was then 80 per cent of theoretical. After removal of the catalyst and unchanged folic acid by filtration under hydrogen, the THFA was precipitated by pouring the glacial acetic acid solution into 500 ml of purified ether². The creamy white precipitate was collected by centrifugation, washed five times with 300 ml portions of ether, and finally dried *in vacuo* over sodium hydroxide. It was stored in darkness under nitrogen in sealed ampules. Steam distillation of an acid solution of this preparation showed that no acetic acid was present. When dried *in vacuo* at 100° for 2 hours over phosphorus pentoxide, the loss of weight was 6 per cent. The dry product yielded 98 per cent of the theoretical nitrogen value by the Kjeldahl method (6) and 105 per cent of the theoretical iodine titration value.

When hydrogenation was continued for 20 hours, 133 per cent of the theoretical hydrogen uptake occurred, and all of the folic acid went into solution. The iodine titration of the final product was 130 per cent of the theoretical value. The nature of the substances formed under these conditions is not known.

* The results of preliminary experiments in this investigation have been reported (1). This investigation has been supported by the Elisabeth Severance Prentiss Fund and by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

¹ The following abbreviations have been used: THFA = 5,6,7,8-tetrahydrofolic acid, DHFA = 7,8-dihydrofolic acid.

² Suggested by Dr L Jaenicke and Dr G R Greenberg.

DHFA was prepared as described by O'Dell *et al* (5) It yielded the theoretical iodine titration value

The hydrogenation of 100 mg of 2-amino-4-hydroxy-6-methylpteridine³ with 100 mg of catalyst in 10 ml of glacial acetic acid was complete in 1 hour The reduced pteridine was recovered after removal of the catalyst by allowing the glacial acetic acid solution to remain *in vacuo* over sodium hydroxide for 1 week The powder thus obtained yielded the theoretical iodine titration value

The hydrogenation of 130 mg of 10-methylfolic acid³ with 130 mg of catalyst in 10 ml of glacial acetic acid was complete in 2 hours The product, recovered by ether precipitation as described for the preparation of THFA, yielded the theoretical iodine titration value

Solutions of the reduced pteridine derivatives were dispensed by syringe from serum bottles filled with hydrogen

Determination of Bound Formaldehyde—A solution containing formaldehyde and the compound to be tested for binding ability was passed through a Dowex 1 chloride⁴ column (1 × 3 cm, 10 per cent cross-linked, 100 to 200 mesh), which was then washed with water until 10 ml of eluate were collected Free formaldehyde is recovered quantitatively by this procedure After incubation with THFA, the formaldehyde is not completely recovered The amount not recovered is referred to as bound formaldehyde In the case of 2-amino-4-hydroxy-6-methyltetrahydropteridine, 2 cm of unground Nuchar C (7) were included on the column to remove material which gave rise to a colored product in the Dowex 1 eluate on standing for several hours in air The Nuchar C does not bind any formaldehyde, nor does it cause the release of formaldehyde bound to THFA

Formaldehyde was determined by the method of Nash (8) in which the acetylacetone reagent was employed

Serine Determination—Serine was determined by a modification of the procedure of Frisell *et al* (9) which involved treatment of the serine with periodate and removal of excess periodate from the reaction mixture with the Dowex 1 columns employed in the determination of bound formaldehyde Formaldehyde arising from the β -carbon of serine is recovered quantitatively, whereas periodate ion is completely retained by the column With this modification, the determination of serine is as sensitive as that of formaldehyde

³ 2-Amino-4-hydroxy-6-methylpteridine and 10-methylfolic acid were obtained through the courtesy of Dr James Smith, Jr, of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

⁴ After removal of the fines the resin was stirred in 1 N HCl for 20 minutes and washed on a column with 2 liters of water

Enzyme Preparations—The preparation and ammonium sulfate fractionation of pigeon liver extract after treatment with Dowex 1 and dialysis have been described previously (2)

Assay of Citrovorum Factor Activity—This assay was carried out essentially by the turbidimetric procedure of Sauberlich and Baumann (10) The assay tubes were inoculated with 0.07 ml of a washed suspension of *Leuconostoc citrovorum* (Klett reading 70, No 660 filter) per 10 ml of medium prior to the addition of the compound to be tested Barium leucovorin⁵ was used as the standard

Radioactivity Determination—Radioactivity was determined on infinitely thin samples as described previously (2)

Protein Determination—Protein was determined by the method of Lowry *et al* (11) with bovine serum albumin as the standard

RESULTS AND DISCUSSION

When THFA was incubated with formaldehyde in equimolar concentration (0.003 M in 0.1 M potassium phosphate, pH 7.6), 77 per cent of the formaldehyde was bound within 45 seconds If the incubation was carried out for 20 minutes, no release of the bound formaldehyde could be detected If the formaldehyde concentration was increased 3-fold, the amount recovered indicated that a maximum of 84 per cent of the THFA reacted The remaining 16 per cent may have been degraded on hydrogenation, yielding products which take up iodine but do not react with formaldehyde

In order to determine the site of formaldehyde binding, various folic acid analogues were tested for this property (Table I) These data suggest that hydrogen atoms on both the 5 and 10 nitrogen atoms of THFA are required for formaldehyde binding, since the compounds lacking either or both of these hydrogens bind formaldehyde poorly A possible structure for the compound formed is shown in Fig 1, this is a derivative of THFA in which nitrogen atoms 5 and 10 are joined by a methylene bridge

The results shown in Table I also reveal that, with the exception of DHFA, compounds which bind formaldehyde poorly cannot stimulate incorporation of formaldehyde into serine The mechanism by which the DHFA preparation catalyzes this incorporation is not clear It is possible that it contains a small amount of THFA Alternatively, DHFA may be converted to THFA by the extract by a dismutation reaction in which equal amounts of THFA and folic acid are formed The conversion of DHFA to THFA has been reported to be catalyzed by xanthine oxidase (12)

⁵ Gift of Dr H P Broquist of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

Although a net increase in free formaldehyde could not be demonstrated after allowing a 0.003 M solution of bound formaldehyde to stand in air at room temperature for 20 minutes, if one mixes bound formaldehyde-C¹⁴ with unlabeled free formaldehyde, radioactivity is released from the

TABLE I
Binding of Formaldehyde by Folic Acid Derivatives

Derivative	Added CH ₂ O recovered (non enzymatic)	Radioactivity in serine (enzymatic)
	<i>per cent</i>	<i>c p m</i>
5,6,7,8-Tetrahydrofolic acid	15	36,000
10-Methyltetrahydrofolic acid	93	450
2-Amino-4-hydroxy-6-methyltetrahydropteridine	95	225
Leucovorin	98	250
Folic acid	99	150
7,8-Dihydrofolic acid	94	3,600

In the non-enzymatic experiments, 3 μ moles of CH₂O were mixed with 4 μ moles of pteridine in open test tubes at 27° in 0.1 M potassium phosphate, pH 7.6, in a total volume of 1 ml. Reaction time, 5 minutes. In the enzymatic experiments, 4 μ moles of formaldehyde-C¹⁴ (104,000 c p m), 20 μ moles of glycine, 4 μ moles of pteridine, and Dowex 1-treated, dialyzed pigeon liver extract equivalent to 20 mg of protein were incubated in a total volume of 1 ml for 10 minutes at 34° in air in 0.1 M potassium phosphate, pH 7.6.

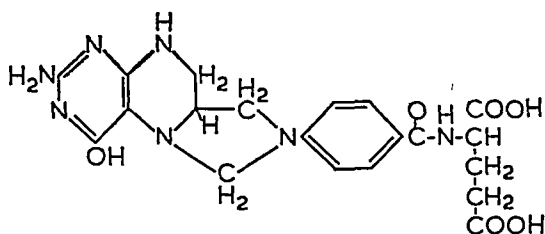


FIG 1 Proposed structure for the product formed by the reaction of formaldehyde with tetrahydrofolic acid

THFA (Table II) This release appears to be promoted by displacement of bound material by that which is free, since as more unlabeled formaldehyde is added, more radioactive formaldehyde is released. The presence of pigeon liver extract did not stimulate the release of bound material.

Although maximal binding occurred at pH 8, the reaction was not greatly influenced by hydrogen ion concentration between pH 5.4 and 8.5. Increasing the pH above 8.5 resulted in a sharp decrease in binding.

Experiments performed previously (13) on the effect of hydrogen ion concentration on formaldehyde incorporation into serine in partially purified pigeon liver extracts showed that the maximal incorporation occurred between pH 7.7 and 8.0 with a sharp drop on either side of these values

TABLE II
Effect of Addition of Unlabeled Formaldehyde on Release of Bound Formaldehyde-C¹⁴

CH ₂ O added	CH ₂ O isolated		Total C ¹⁴ released	
	With enzyme	Without enzyme	With enzyme	Without enzyme
<i>μmoles</i>	<i>μmoles</i>	<i>μmoles</i>	<i>c p m</i>	<i>c p m</i>
0	0.12	0.06	1,600	2,500
2	1.20	1.18	8,700	10,200
5	2.97	3.30	14,400	15,400
10	7.26	8.00	18,700	18,300
15	12.11	13.30	22,400	20,400

Bound formaldehyde-C¹⁴ containing 3.8 μ moles of THFA and 2.5 μ moles of formaldehyde-C¹⁴ (65,000 c p m) was incubated in a total volume of 1 ml for 10 minutes at 34° in open test tubes in 0.1 M potassium phosphate, pH 7.6. The enzymatic experiments contained, in addition, Dowex 1-treated, dialyzed pigeon liver extract equivalent to 15 mg of protein. At the end of the incubation the mixture was poured through Dowex 1 columns as described under "Materials and methods." Protein was removed by adding 1 ml of 20 per cent trichloroacetic acid to the eluate obtained from the enzymatic experiments. After determination of the formaldehyde in these eluates, carrier formaldehyde was added and precipitated as the dimedon derivative. The radioactivity values are calculated, assuming 100 per cent recovery of the added CH₂O. In order to show that the enzyme preparation was active, an identical incubation was carried out in the absence of unlabeled formaldehyde but with 20 μ moles of glycine added. 20,700 c p m were incorporated into serine.

The results shown in Table III demonstrate that the rate of incorporation of free and bound formaldehyde into serine is approximately the same. If the bound formaldehyde is intermediate in the incorporation of formaldehyde into serine, one would expect that the rate of its incorporation would be at least as fast as the rate at which free formaldehyde is incorporated.

The possibility that the bound formaldehyde is converted to free formaldehyde prior to its condensation with glycine has been investigated. Bound radioactive formaldehyde was incubated with pigeon liver extract supplemented with glycine in the presence of unlabeled formaldehyde. The specific activity of the serine formed was determined at various times.

intervals as shown in Table IV. If all of the bound formaldehyde was released before incorporation into serine, the maximal specific activity of the serine would be 4600 c p m per μ mole. The decrease in the specific activity of the serine with time is presumed to result from the incorporation of unlabeled formaldehyde into serine via free THFA. This experiment demonstrates that bound formaldehyde can be converted to the serine β -carbon without being released from THFA.

Experiments on the ability of bound formaldehyde to support the growth of *L. citrovorum* have shown that it is 1/1300 as active as leucovorin on a molar basis. THFA under the same conditions was 1/2000 as active

TABLE III

Comparison of Rate of Serine Formation from Free and Bound Formaldehyde

Time	Total activity of serine	
	From bound CH_2O	From free CH_2O
<i>min</i>	<i>c p m</i>	<i>c p m</i>
0 5	2,000	1,700
1 0	2,850	2,000
2 0	4,300	3,600
5 0	8,000	7,000
8 0	10,900	10,400

Dowex 1-treated, dialyzed pigeon liver extract equivalent to 12 mg of enzyme protein, 20 μ moles of glycine, 7 μ moles of THFA, and 3.4 μ moles of formaldehyde C^{14} (88,400 c p m) were incubated in a total volume of 1 ml at 34° in open test tubes in 0.1 M potassium phosphate, pH 7.6. In the bound formaldehyde experiment, the THFA and formaldehyde were incubated together 5 minutes prior to addition to the enzyme and glycine. In the free formaldehyde experiment, the THFA was included with the enzyme and the glycine. The formaldehyde was added last.

as the standard compound. The THFA-formaldehyde mixture did not inhibit the growth response of this organism to leucovorin.

The extent of the conversion of bound formaldehyde- C^{14} to serine β -carbon is shown in Table V. After prolonged incubation and the addition of fresh enzyme, a maximum of 63 per cent of the added formaldehyde was incorporated into serine. Most of the remainder of the added radioactivity could be recovered as free formaldehyde after acid treatment of the incubation mixture. Deodhar⁶ has observed the conversion of 80 per cent of added formaldehyde to serine in an enzyme preparation extracted from rat liver mitochondria. These results indicate that, if 50 per cent of the bound formaldehyde is associated with the D form of

⁶ Deodhar, S. D., personal communication.

TABLE IV
*Specific Activity of Serine Formed from Bound Formaldehyde-C¹⁴
 in Presence of Unlabeled Formaldehyde*

Incubation time	Specific activity of serine
<i>min</i>	<i>c p m per μmole</i>
0 5	24,000
1 0	23,000
2 0	22,000
5 0	15,000

Four solutions containing 39.5 μ moles of unlabeled formaldehyde, 98 μ moles of glycine, 198 μ moles of potassium phosphate, pH 8, and Dowex 1-treated, dialyzed pigeon liver extract equivalent to 120 mg of protein were incubated at 34° in 2 \times 17 cm test tubes for 5 minutes in a total volume of 4 ml. During this time a THFA-C¹⁴H₂O solution was prepared containing 8.5 μ moles of C¹⁴H₂O (26,000 c p m per μ mole), 16 μ moles of THFA, and 150 μ moles of potassium phosphate, pH 8, per ml. 1 ml of this mixture was pipetted into each of the enzyme solutions, and the incubation was carried out for the appropriate time interval. Each incubation was terminated by freezing the mixture in a dry ice-Cellosolve bath. 3 ml of 20 per cent trichloroacetic acid were then added, and the solution was thawed. Serine was isolated from the protein-free supernatant solution by chromatography on Dowex 50, and the serine was determined as described. The results shown are the average of two serine determinations.

TABLE V
Extent of Conversion of Bound Formaldehyde-C¹⁴ to Serine

Incubation time	Activity in serine	Activity of recovered CH ₂ O	Radioactivity recovered
<i>min</i>	<i>c p m</i>	<i>c p m</i>	<i>per cent</i>
30	50,000	40,000	96
60	54,000	34,000	94
120	54,000	32,000	92
120*	58,000	22,600	87
180†	60,000	19,800	85

3.6 μ moles of formaldehyde-C¹⁴ (94,000 c p m), 4.0 μ moles of THFA, 20 μ moles of glycine, 10 mg of enzyme protein (24 to 47 per cent ammonium sulfate fraction of pigeon liver extract), and 1 μ mole of pyridoxal phosphate were incubated under hydrogen in Thunberg tubes at 34°. The reaction was stopped by adding 1 ml of 20 per cent trichloroacetic acid solution. Unchanged formaldehyde-C¹⁴ was isolated from the water wash of the Dowex 50 columns used to isolate serine from the protein-free supernatant solution as the dimedon derivative.

* After incubation for 60 minutes, an additional 10 mg of enzyme protein were added, and the incubation was continued for 60 more minutes.

† After incubation for 120 minutes, an additional 10 mg of enzyme protein were added, and the incubation was continued for 60 more minutes.

THFA and 50 per cent with the L form, a pathway must exist for the conversion to serine of some of the formaldehyde bound to the unnatural form. This could occur by a direct transfer of a formaldehyde unit from one of the forms of THFA to the other. A compound involving a methylene bridge between the two isomers of THFA could be an intermediate in this process. The possibility that the biologically inactive form might release its formaldehyde, which could subsequently condense with the active isomer of THFA, has not been excluded. It is also possible that the active form of THFA is formed in a predominant amount in the reduction of folic acid, or that both isomers are biologically active.

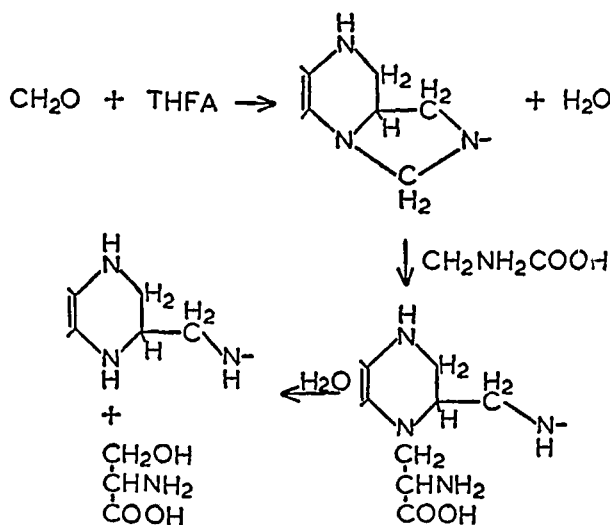


Fig 2 Suggested outline of the pathway of formaldehyde incorporation into serine

Paper chromatography at 3° of a mixture of formaldehyde- C^{14} and THFA on Whatman No 1 filter paper, with 0.1 M potassium phosphate at pH 8, as the solvent revealed two radioactive spots (R_F 0.27 and R_F 0.41) containing approximately the same amount of radioactivity. It is possible that these represent diastereoisomers, since THFA contains a second asymmetric center in the L-glutamic acid moiety. Two radioactive peaks could also be obtained if a mixture of formaldehyde- C^{14} and THFA was chromatographed on Whatman standard grade cellulose with the same solvent. Only about 60 per cent of the added radioactivity was recovered in these two compounds. Further work is required to determine the relationship of these two compounds to each other and to the two formaldehyde derivatives of THFA formed on the breakdown of serine in the rat liver system described by Deodhar *et al* (14).

One might visualize the conversion of formaldehyde to serine as shown in Fig 2.

The first step would be the non-enzymatic condensation of formaldehyde with THFA to form a methylene bridge compound between positions 5 and 10. This compound has been suggested (3) as a possible structure for the active formaldehyde derivative. The fact that both the 5 and 10 nitrogen atoms must have free hydrogen atoms in order that tetrahydropteridine derivatives may retain their ability to bind formaldehyde supports this suggestion. The next step would be the condensation of this derivative with glycine which has been activated by the serine hydroxymethylase (2) enzyme which possesses pyridoxal phosphate as its cofactor (15, 16). It is not known whether the formaldehyde remains bound to the THFA through the 5 or 10 nitrogen atom in the derivative thus formed. In either case, hydrolysis of this derivative would give rise to serine and the free pteridine.

Recent work by Jaenicke (17) has indicated that a mixture of formaldehyde and THFA is rapidly oxidized to 10-formyl-5,6,7,8-tetrahydrofolic acid by a pigeon liver system requiring triphosphopyridine nucleotide. It is likely that the active compound formed in the mixture is the same as the compound active for serine β -carbon formation.

The author is indebted to Dr. Warwick Sakami and Dr. Merton F. Utter for much helpful advice.

SUMMARY

It has been shown that formaldehyde reacts rapidly and non-enzymatically with 5,6,7,8-tetrahydrofolic acid to form one or more compounds which are active precursors of the β -carbon of serine.

Both the 5 and 10 positions of the reduced pteridine appear to be involved in formaldehyde binding and serine formation.

A modification of a published method of serine determination is described which makes this procedure as sensitive as that for formaldehyde.

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BACTERIAL FERMENTATION OF NICOTINIC ACID

I END PRODUCTS

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The ability of certain bacterial species to oxidize nicotinic acid was first reported by Allson (1) who used suspensions of bacteria isolated by Dubos and Miller (2). Koser and Baird (3) later isolated strains of *Pseudomonas fluorescens* which grew on a synthetic medium in which nicotinic acid was the sole carbon source. These strains did not attack isonicotinic acid, picolinic acid, or pyridine. Nichol and Michaelis (4) then demonstrated that the destruction of nicotinic acid by this organism was an oxidative process accompanied by an uptake of oxygen. Most recently Hughes (5), using the same organism, has presented evidence that 6-hydroxynicotinic acid is an intermediate in oxidative degradation of nicotinic acid.

There has been no previous study of the degradation of nicotinic acid by anaerobic bacteria. By means of the enrichment culture technique an anaerobic spore-forming rod has been isolated which ferments nicotinic acid and which utilizes high concentrations of this compound for its growth. The dependence on a high concentration of nicotinic acid for anaerobic growth suggests that the compound may serve as an energy, carbon, or nitrogen source for growth. This report presents evidence showing that the fermentation of nicotinic acid results in the formation of acetate, propionate, ammonia, and CO_2 . The anaerobic bacteria reported here may provide a system for the investigation of the mechanism of the direct utilization of the energy of nicotinic acid and of the nature of the intermediates involved. A preliminary report (6) of this study has been published.

Materials and Methods

Isolation Procedure—The nicotinic acid-degrading organism was isolated from mud of the Potomac River. A small amount of mud was suspended in 50 ml of a solution containing 0.5 per cent nicotinic acid and 0.03 per cent $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ in a ground glass-stoppered bottle. Precautions were taken to exclude air. After 3 weeks incubation at 37° some turbidity was noted. The culture was mixed thoroughly and 5 ml were

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transferred to a solution of 0.5 per cent nicotinic acid, 0.03 per cent $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.2 per cent peptone, 0.2 per cent yeast extract, 0.05 M phosphate buffer at pH 7.4, and a salt solution made up of 0.002 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0001 per cent $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.0001 per cent $\text{FeCl}_3 \cdot 7\text{H}_2\text{O}$. The mixture was incubated at 37° until maximal turbidity was reached, at which time 50 to 80 per cent of the nicotinic acid had disappeared. A culture was obtained after three serial transfers in the above medium which was relatively free of impurities from the mud. The utilization of nicotinic acid was followed by noting the disappearance of absorption characteristic of nicotinic acid at $260 \text{ m}\mu$. By means of dilution in anaerobic agar shake cultures several colonies were isolated.

Preparation of Cells—The bacteria were grown in 20 liter bottles in media containing 0.4 per cent nicotinic acid, 0.2 per cent peptone, 0.6 per cent yeast extract, 0.05 M phosphate buffer at pH 7.4, the salt solution previously described, and 700 mg of $\text{Na}_2\text{S}_2\text{O}_4$. After incubation at 37° for 3 days the cells were harvested in a Sharples centrifuge and washed twice with 10 volumes of ice-cold, 0.03 per cent $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ and then lyophilized and stored at -15° . Cell suspensions were prepared with 100 mg of bacteria per ml of ice-cold distilled H_2O .

Growth Characteristics—The ability of this organism to grow on varying concentrations of nutrient was determined by measuring with a Klett colorimeter the density of growing cultures at various time periods.

Separation and Identification of Acid Products—The volatile acids produced during growth and during incubation with resting cell suspensions were separated from the acidified reaction mixture by steam distillation in a Markham type still (7). An aliquot was titrated and chromatographed on Whatman No. 3 filter paper, washed with oxalic acid, and developed with butanol- H_2O -diethylamine (100:15:1) (8).

The per cent of each acid in the acid mixture was also determined by Duclaux distillation. Further identification was achieved by separation of the acid mixture on a silica gel column according to the method of Elden (9). The separated acids were chromatographed on paper and also determined by Duclaux distillation. Acetic acid was also identified as the sodium uranyl salt (10).

Nicotinic acid was determined by the method of Hughes and Williamson (11) and ammonia by nesslerization.

Results

Colony Form and Morphology—Of the colonies isolated from the anaerobic shake culture one contained a pure culture of an anaerobic bacterium that could degrade nicotinic acid. This organism produced small, disk-shaped, smooth, translucent, and colorless colonies. The bacterium

is a small motile rod containing terminal spores and thus belongs in the genus *Clostridium*. No further identification has been made.

Growth Requirements—The composition with respect to peptone and yeast extract was varied as shown in Table I. Within the limits of concentrations used 0.2 per cent peptone and 0.6 per cent yeast extract gave the best growth and this level was chosen for large scale preparations. Casein hydrolysate would not serve as a substitute for peptone and the effect of yeast extract could not be reproduced by a vitamin mixture containing 10 γ each, per ml. of medium, of biotin, calcium pantothenate, pyridoxamine, folic acid, vitamins A and D, thiamine, tocopherol, inositol,

TABLE I

Optimal Concentration of Yeast Extract and Peptone for Growth

The medium otherwise contains 0.03 per cent Na_2S , 9H₂O, 0.05 M phosphate buffer, 0.5 per cent nicotinic acid, and 1 per cent of a salt solution made up of 0.002 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0001 per cent $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.0001 per cent $\text{FeCl}_3 \cdot 7\text{H}_2\text{O}$.

Peptone, final concentration	Yeast extract, final concentration	Growth after 96 hrs., density
<i>per cent</i>	<i>per cent</i>	<i>Klett reading</i>
0.2		25
0.4		25
0.6		47
0.2	0.2	47
0.4	0.2	71
0.6	0.2	68
0.2	0.4	97
0.2	0.6	141
	0.2	33
	0.4	32
	0.6	99

riboflavin, *p*-aminobenzoic acid, nicotinamide, and ascorbic acid. The high level of yeast extract used indicates that the factor responsible may be acting as a substrate. A preliminary examination of other possible substrates for this bacteria showed that glucose is by far the most effective compound for its growth, yielding 2 to 3 times more bacteria than does nicotinic acid. Pyruvic acid and nicotinamide achieved the same growth response as nicotinic acid, while DL-alanine, β -alanine, picolinic acid, isonicotinic acid, and *N*-methylnicotinamide were ineffective. The ability of glucose to support growth suggests the possibility that the ability to degrade nicotinic acid is adaptive and this point is now under investigation.

By using 0.6 per cent yeast extract and 0.2 per cent peptone, which had been determined as the best concentrations for growth in the presence of nicotinic acid, the concentration of nicotinic acid was varied. As indi-

cated in Fig 1 there was no growth in the absence of nicotinic acid. There is increasing growth with increasing concentrations of nicotinic acid reaching a maximum at 0.4 per cent.

End Products—In a 2 liter growing culture incubated for 7 days at 37° it was found that 156 μ moles of nicotinic acid had disappeared and 360 μ moles of steam-volatile acid were formed. For each acid equivalent of

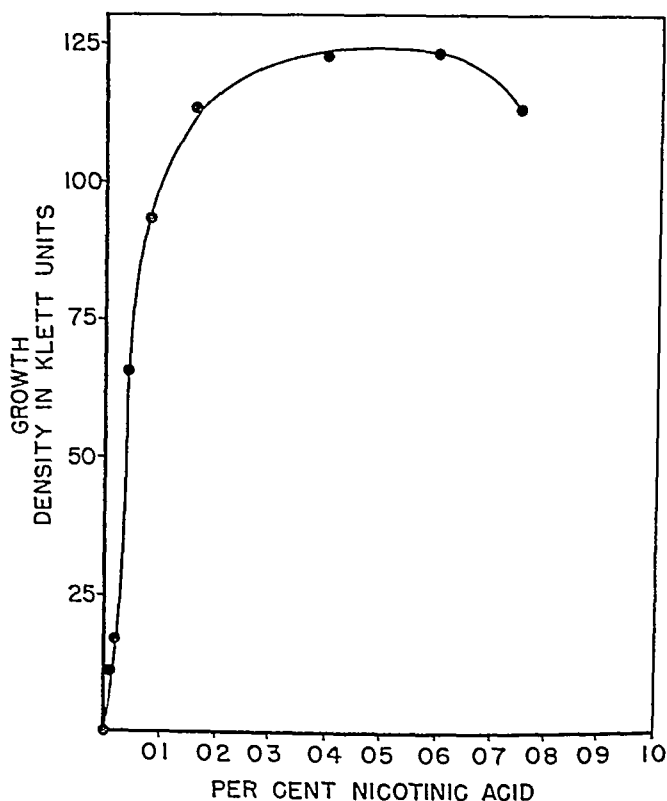


FIG 1 The effect of nicotinic acid on anaerobic growth as measured by the density of the cultures

nicotinic acid disappearing, approximately 2 acid equivalents of steam-volatile acid were thus formed.

With resting cell suspensions nicotinic acid is fermented without yeast extract, peptone, or the salt mixture. As shown in Table II, when nicotinic acid is incubated with a resting cell suspension in a bicarbonate buffer system with 95 per cent nitrogen-5 per cent CO_2 as the gas phase (pH 7.4), 1 mole each of propionic acid, acetic acid, and ammonia is formed for each mole of nicotinic acid that disappears. There is also a net formation of 1 mole of acid per mole of nicotinic acid fermented, as measured by the evolution of CO_2 from the bicarbonate buffer (see also the data of Table III). Since the conversion of nicotinic acid to acetic acid, propionic acid, and

ammonia should not lead to the observed net increase in acidity, an additional acid product must be formed. This product has been identified as CO_2 . The data of Table IV show that the fermentation of nicotinic acid

TABLE II
End Products of Nicotinic Acid Fermentation

Compound	μmoles
Nicotinic acid disappeared	131
Ammonia formed	122
Acetic acid formed*	110
Propionic acid formed*	107
Net acid production†	109

The incubation flask contained 131 μmoles of nicotinic acid, 0.025 M KHCO_3 , 5.6 ml of bacterial suspension (30 mg per ml), and water to a volume of 28 ml. Incubated in an atmosphere of 5 per cent CO_2 -95 per cent nitrogen, at 36°, until gas production ceased. Nicotinic acid was determined by the cyanogen bromide method (11), ammonia by nesslerization, propionic and acetic acids by Duclaux distillation, silica gel separation, and identified by paper chromatography, and acid production was measured by CO_2 release from bicarbonate buffer. All values corrected for endogenous metabolism.

* A total of 345 μmoles of steam-volatile acids, composed of acetic and propionic acids, was produced. A comparable cell suspension incubated without nicotinic acid produced 128 μmoles of acetic acid.

† The endogenous production of net acid was 37 μmoles .

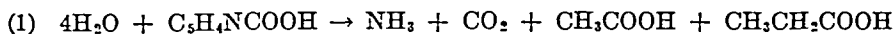
TABLE III
 CO_2 Production

The additions are as follows: 20 mg of washed cell suspension, 200 μmoles of buffer, pH 6.0, nicotinic acid as indicated, H_2O to 2 ml final volume. Gas N_2 , temperature 26°. Reaction started with the tipping of the bacteria and continued to cessation of gas production. In a comparable vessel incubated without nicotinic acid 3.0 μmoles of CO_2 were formed. Figures given corrected for endogenous metabolism.

Nicotinic acid added	CO_2 produced
μmoles	μmoles
0.94	0.82
1.89	1.65
3.76	3.25

in a bicarbonate-free medium (pH 6.0) results in the release of 1 mole of CO_2 per mole of nicotinic acid added.

The results of the above experiment may be formulated according to the following equation:



Specificity—Various compounds related structurally to nicotinic acid were tested for acid production activity. Pyridine, picolinic acid, iso nicotinic acid, quinolinic acid, isocinchomeionic acid, anthranilic acid, *p*-aminobenzoic acid, 3-hydroxyanthranilic acid, kynurenic acid, and *N*-methylnicotinamide were inactive. Nicotinamide, on the other hand, was found to be as active as nicotinic acid.

TABLE IV
Acid Production

The additions are as follows: 20 mg of washed cell suspension, 50 μ moles of KHCO_3 , nicotinic acid as indicated, H_2O to 2 ml final volume. Gas 5 per cent CO_2 in N_2 , temperature 26° . Reaction started with the tipping of the bacteria and continued to cessation of gas production. In a comparable vessel incubated without nicotinic acid 1.6 μ moles of acid were formed. Figures given corrected for endogenous metabolism.

Nicotinic acid added	Acid produced
μ moles	μ moles CO_2
0.94	0.94
1.89	1.88
3.76	3.20
5.65	4.70
7.50	6.15

DISCUSSION

The role nicotinic acid plays in the growth of the organism isolated is open to speculation. The high concentration needed for maximal growth points to its role as a substrate serving as an energy, carbon, or nitrogen source for growth. The possibility exists that a series of intermediate oxidation-reduction reactions takes part in the process. This would most certainly be the case, if as seems possible, the organism derives energy for growth from this fermentation.

Very little may be said about the steps in the degradation from a consideration of the end products, however, if one assumes that these compounds are not reformed from smaller components, the products place a limit on the possibilities. The observation that pyridine will not act as a substrate indicates that the decarboxylation of nicotinic acid is not the first step.

On the basis of these facts and other known reactions, two routes for the degradation of nicotinic acid may be postulated as shown in Fig. 2.

One may postulate, as in Route A, a cleavage between the nitrogen atom and carbon 2 or 6 and between carbon atoms 3 and 4 to yield intermediates

that may be directly converted to propionic and malonic acids, malonic acid may then be decarboxylated to acetic acid and CO_2 (12). A cleavage between the nitrogen atom and carbon atom 2 or 6 and between carbon atoms 4 and 5 would yield compounds which could be converted to acetic and methylmalonic acids. Methylmalonic acid is known to be decarboxylated to propionic acid and CO_2 in other organisms (13). Evidence is presented elsewhere which suggests the intermediary formation of 6-hydroxynicotinic acid in the degradation of nicotinic acid (14). This is further support for the hypothetical cleavage between the nitrogen atom and carbon 6.

The specificity studies show that only nicotinic acid and nicotinamide are active in supporting growth of this organism. That the latter compound is active suggests the presence of an enzyme which hydrolyzes nico-

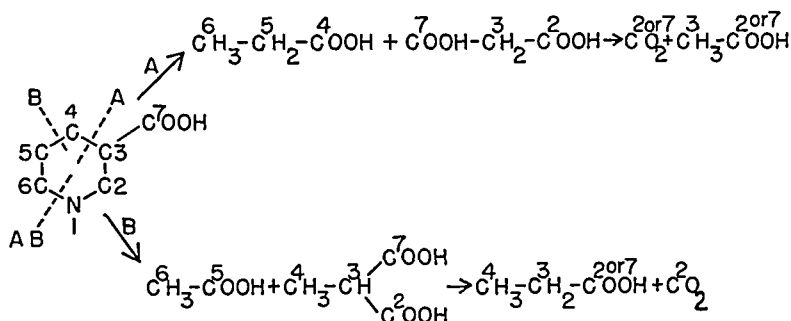


FIG. 2 Possible routes of nicotinic acid degradation

tinamide to nicotinic acid and ammonia. None of the intermediates, such as kynurenic, anthranilic, 5-hydroxyanthranilic, and quinolinic acids, which have been shown to give rise to nicotinic acid in mammalian tissue, is fermented.

SUMMARY

An anaerobic bacterium has been isolated from the soil which ferments nicotinic acid to propionic acid, acetic acid, carbon dioxide, and ammonia.

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BACTERIAL FERMENTATION OF NICOTINIC ACID*

II ANAEROBIC REVERSIBLE HYDROXYLATION OF NICOTINIC ACID TO 6-HYDROXYNICOTINIC ACID

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As previously reported (1-3), an anaerobic bacterium has been isolated from soil which ferments nicotinic acid (NA) according to the following equation



Although the over-all reaction requires no external energy source, the nature of the end products indicates that at least two oxidation-reduction steps occur during the reaction. It was of interest, therefore, to test the effect of an oxidizing agent on the fermentation of NA. One may in this manner force the accumulation of a more oxidized compound which may be an intermediate in the fermentation or may be derived from an intermediate. The oxidation of NA by methylene blue to 6-hydroxynicotinic acid (HNA), catalyzed by a nicotinic acid-fermenting organism, is the subject of this report.

EXPERIMENTAL

Methylene blue oxidations were performed in Thunberg tubes containing methylene blue and nicotinic acid in the side arm and bacteria and buffer in the main space. Before tipping, the tubes were kept in an ice bath and evacuated for 5 minutes with frequent tapping to release trapped air. The reaction was started with tipping in of the methylene blue and the substrate and terminated, after complete decolorization, by boiling the reaction mixture for 5 minutes. The same procedure, except for the omission of methylene blue, was carried out in the fermentation experiments. In this case the incubation time was fixed at 1 hour.

Analytical Methods—The supernatant fluid of the reaction mixture was used directly for chromatography. For the radioisotope experiments 0.1 ml of the supernatant fluid was streaked on washed Whatman No. 3 paper and chromatographed in a mixture of methyl ethyl ketone, *tert*-butanol,

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diethylamine, and H_2O (40 40 1 20) The radioactive bands were eluted with 0.05 N NaOH The eluates were collected and adjusted to a final volume of 2 ml Aliquots were used for determination of concentration in the Beckman spectrophotometer and for radioisotope determination A small blank from the paper was used for correction in the determination of the concentration

Determination of the stoichiometry of this reaction was made possible by the quantitative elution of the acids from the paper chromatograms and the determination of their concentrations from their absorption at 260 $\text{m}\mu$ The absorption coefficient of NA and HNA at 260 $\text{m}\mu$ in 1 N HCl is, respectively, 5.2×10^3 and 1.26×10^4 The absorption coefficient of methylene blue at 660 $\text{m}\mu$ at pH 3.5 is 7.4×10^4 (4)

Radioisotope Determination—Disks of paper weighing 2 mg per cm^2 were centered in metal planchets to which was added 0.1 ml of 0.1 per cent agar solution After drying under infrared lamps, no more than 0.05 ml of the sample to be counted was introduced on the paper at one time and then dried They were counted with a thin window Geiger-Muller tube A correction factor for the weight of paper and sample was used according to Libby (5)

Ion Exchange Chromatography—HNA and NA were also separated by ion exchange chromatography with IRA-400 in a column 30 cm long by 1.3 cm in diameter The resin was prepared by cycling with 2 N NaOH and 2 N HCl until the absorption at 260 $\text{m}\mu$ reached a minimal constant value, washed with 2 N sodium formate until the eluates were free of chloride ion, and then washed with 200 ml of distilled H_2O The sample containing neutralized NA and HNA was introduced in 10 ml of H_2O , washed through with 100 ml of H_2O , and developed with 0.28 M sodium formate made alkaline to pH 10.5 Fractions of 10 ml each were collected at the rate of 1 ml a minute until all the NA was eluted After the elution of NA several more fractions were collected and then the developing solution was changed to 2 N formic acid for elution of the HNA The concentration of the acids was determined by their absorption at 260 $\text{m}\mu$ NA was easily differentiated from HNA by the fact that HNA has a high absorption at 290 $\text{m}\mu$ and NA has little The fractions containing NA and HNA were pooled separately, acidified with H_2SO_4 , and steam-distilled to remove the formic acid Each sample was then extracted with ether, evaporated to dryness, and taken up in H_2O

Materials—Radioactive nicotinic acid was a gift from Alan H. Mehler 6-Hydroxynicotinic acid was obtained from H. Light Company, Ltd., London

Results

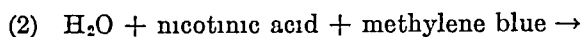
Methylene Blue Oxidation of Nicotinic Acid—10 μ moles of NA were incubated with 10 μ moles of methylene blue, 40 mg of lyophilized bacteria suspended in 0.03 per cent $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, and 300 μ moles of potassium phosphate buffer, pH 7.4, *in vacuo*, at 25° in a Thunberg tube. Within 2 hours all of the methylene blue was decolorized. After inactivation of the bacteria, and centrifugation, an aliquot of the supernatant fluid of the reaction mixture was chromatographed on paper with water-saturated butanol and formic acid (95:5) as the developing solvent. The ultraviolet-absorbing spot corresponding to NA, $R_F = 0.80$, disappeared and a new spot with an R_F of 0.70 appeared, indicating that NA had been transformed to a new compound by the action of methylene blue, catalyzed by the bacteria. The new spot was eluted from the paper and the ultraviolet absorption spectrum proved to correspond to the spectrum of 6-hydroxynicotinic acid which has been shown by Hughes (6) to be a metabolite in *Pseudomonas fluorescens*.

A larger scale experiment with 2.5 gm of lyophilized bacteria and 0.5 mmole of NA was set up. The supernatant fluid was concentrated *in vacuo* to a small volume, then streaked on solvent- and alkali-washed Whatman No. 3 paper, and chromatographed with a mixture of methyl ethyl ketone, *tert*-butanol, diethylamine, and H_2O (40:40:1:20) as the developing solvent. The band corresponding to HNA was eluted, neutralized, and evaporated *in vacuo* to dryness. The residue was dissolved in a small volume of hot distilled H_2O and a small amount of Norit was added. After filtration the HNA crystallized upon cooling. The material was crystallized three more times and yielded 20 mg of crystals that melted at 308–309° (uncorrected). Authentic HNA also melted at 308–309° (uncorrected) and a mixed melting point of the experimental and authentic sample showed no melting point depression. The absorption spectrum of the experimental sample was directly superimposable on that of an authentic sample over most of the range (Fig. 1).

Oxidation of C^{14} -Carboxyl-Labeled NA—To establish conclusively that the HNA isolated from the bacteria was formed from NA oxidation, C^{14} -carboxyl-labeled NA was incubated with two concentrations of methylene blue. In Table I it may be seen that radioactive HNA was formed in both experiments and, within experimental error, had the same specific radioactivity as the starting NA.

Stoichiometry—In order to determine the stoichiometry of the reaction, 5.1 μ moles of NA were incubated with 1.87 μ moles of methylene blue and 40 mg of bacteria. After complete decolorization of the methylene blue it was found that 3.4 μ moles of NA remained and 2.01 μ moles of HNA were

formed. These results and those for Experiment 2 in Table I demonstrate a stoichiometric conversion of the reactants to the products. Thus the reaction may be formulated by the following equation:



6-hydroxynicotinic acid + reduced methylene blue

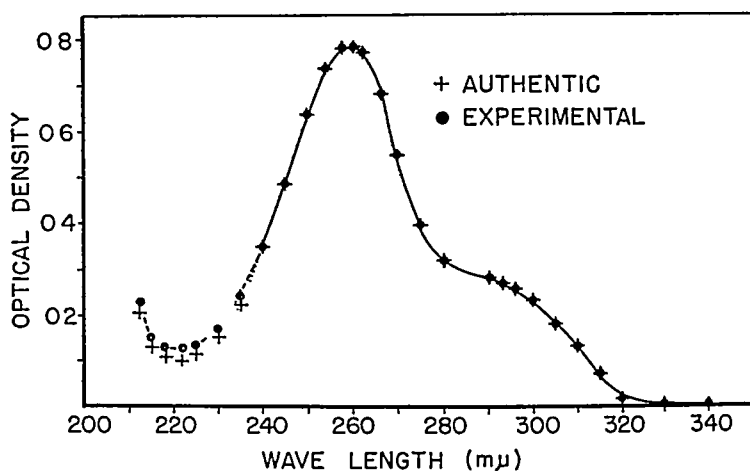


Fig. 1. Absorption spectrum of 6-hydroxynicotinic acid in 1 N HCl.

TABLE I

Oxidation of Radioactive Nicotinic Acid

The additions are as follows: 5.1 μ moles of nicotinic acid (specific activity = 774 c.p.m. per γ), 300 μ moles of potassium phosphate buffer, pH 7.4, 40 mg of bacteria, methylene blue 3.74 μ moles in Experiment 2 and 1.87 μ moles in Experiment 3. Final volume = 3.0 ml, temperature = 25°. Reaction started with tipping in of methylene blue and substrates and terminated, at completion of decolorization of methylene blue, by boiling for 5 minutes.

	Experiment 1	Experiment 2		Experiment 3	
	NA (5.1)*	NA (1.3)*	HNA (3.6)*	NA (3.4)*	HNA (2.01)*
	c.p.m. per μ mole	c.p.m. per μ mole	c.p.m. per μ mole	c.p.m. per μ mole	c.p.m. per μ mole
Specific activity	96,500	94,700	97,000	98,000	88,000

* Micromoles at the end.

Formation of HNA during Fermentation—It was of interest to determine whether, during the fermentation of NA, formation of HNA could be detected. An experiment was performed with C^{14} -carboxyl-labeled NA in the absence of methylene blue. The conditions and the results are listed in Table II. Experiment 1, Table II, was a zero time control in which all additions were made and the reaction mixture was immediately inactivated.

by boiling for 5 minutes. It was then incubated at the same temperature and for the same length of time as the other experiments. In Experiment 2, in which only radioactive NA was added initially, a small amount of labeled HNA was formed. In Experiment 3, in which both labeled NA and unlabeled HNA were incubated together, the reisolated acids had almost equal specific radioactivities. The presence of unlabeled HNA during the incubation increased the amount of labeled HNA formed and led to a lowering of the specific radioactivity of NA. These results may be explained by assuming that the conversion of NA to HNA is reversible. Unlabeled HNA may act as an oxidizing agent forming, from radioactive NA, radioactive HNA. In turn the non-radioactive HNA may be re-

TABLE II

Oxidation of NA to HNA during Fermentation of NA

The additions are as follows: 2 μ moles of NA (specific activity = 600 c p m per γ), 5 μ moles of HNA as indicated, 300 μ moles of potassium phosphate buffer, pH 7.4, 40 mg of bacteria. Experiment 1 is a complete system inactivated at zero time. Experiment 2 contained NA during incubation and HNA added after inactivation. Experiment 3 contained both NA and HNA during incubation. Final volume = 3.0 ml, temperature = 25°, incubation time = 1 hour. Reaction started with tipping in of substrates and ended with boiling for 5 minutes.

	Experiment 1		Experiment 2		Experiment 3	
	NA	HNA	NA	HNA	NA	HNA
	c p m per μ mole	c p m per μ mole	c p m per μ mole	c p m per μ mole	c p m per μ mole	c p m per μ mole
Specific activity	2.8×10^6	0	1.75×10^6	1.45×10^6	7.26×10^5	6.3×10^5

duced to non-radioactive NA, thus reducing the specific radioactivity of the NA. It may be readily perceived that under these conditions the concentration of HNA and NA would not be altered despite the change in concentration of labeled compounds. With this bacterial preparation very little fermentation occurred and the concentration of the acids did not appreciably change.

Radioactive NA and HNA from these experiments were chromatographed in eight different solvent systems and the position of the experimentally produced HNA was found to correspond to that of an authentic sample (Table III). The radioactivity, determined by cutting the paper chromatograms into 1 cm strips and counting them with an ultrathin window Geiger-Müller tube, was directly superimposable on the ultraviolet-absorbing spots. No radioactivity or ultraviolet-absorbing spots were found anywhere else on the paper. A radioautograph demonstrated the same results (Fig. 2).

formed. These results and those for Experiment 2 in Table I demonstrate a stoichiometric conversion of the reactants to the products. Thus the reaction may be formulated by the following equation

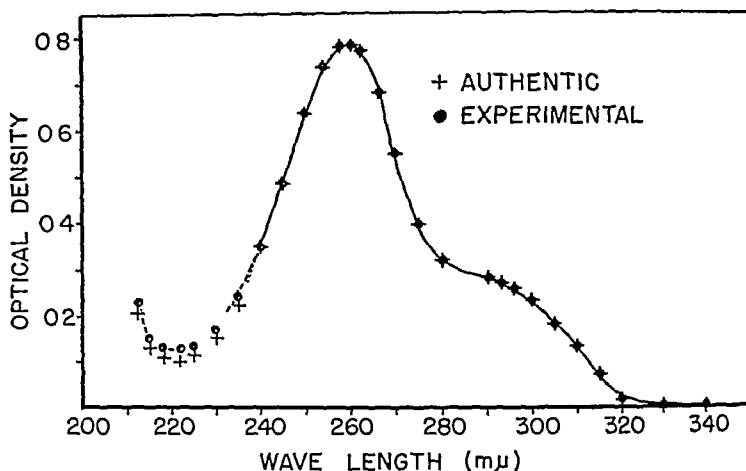
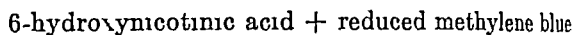
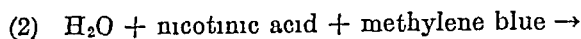


FIG 1 Absorption spectrum of 6-hydroxynicotinic acid in 1 N HCl

TABLE I

Oxidation of Radioactive Nicotinic Acid

The additions are as follows: 5.1 μ moles of nicotinic acid (specific activity = 96 c p m per γ), 300 μ moles of potassium phosphate buffer, pH 7.4, 40 mg of bacteria, methylene blue 3.74 μ moles in Experiment 2 and 1.87 μ moles in Experiment 3. Final volume = 3.0 ml, temperature = 25°. Reaction started with tipping in of methylene blue and substrates and terminated, at completion of decolorization of methylene blue, by boiling for 5 minutes.

	Experiment 1	Experiment 2		Experiment 3	
	NA (5.1)*	NA (1.3)*	HNA (3.6)*	NA (3.4)*	HNA (2.0)*
Specific activity	c p m per μ mole 96,500	c p m per μ mole 94,700	c p m per μ mole 97,000	c p m per μ mole 98,000	c p m per μ mole 88,000

* Micromoles at the end

Formation of HNA during Fermentation—It was of interest to determine whether, during the fermentation of NA, formation of HNA could be detected. An experiment was performed with C^{14} -carboxyl-labeled NA in the absence of methylene blue. The conditions and the results are listed in Table II. Experiment 1, Table II, was a zero time control in which all additions were made and the reaction mixture was immediately inactivated

Formation of NA from HNA—Radioactive HNA was isolated from the previous experiments and used to demonstrate the reversibility of the reaction. In Table IV are the results of such an experiment. Experiment 1 was a zero time control and contained both radioactive HNA and unlabeled NA. Experiment 2 contained only labeled HNA. Experiment 3 contained labeled HNA and unlabeled NA during the incubation period. The formation of NA from HNA demonstrates the reversal of the reaction. Only in Experiment 3 was there appreciable formation of radioactive NA, indicating that NA was necessary for the reaction to proceed. Here, too, there was no appreciable change in the concentration of reactants. In order to check the results of the paper chromatographic separa-

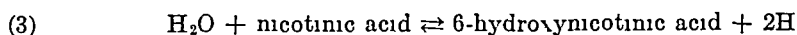
TABLE IV
Reduction of HNA to NA

The additions are as follows: 2 μ moles of HNA (specific activity 1.56×10^5 c p m per μ mole), 2 μ moles of NA, 300 μ moles of potassium phosphate buffer, pH 7.4, 40 mg of bacteria. Experiment 1 is a complete system inactivated at zero time by boiling for 5 minutes. Experiment 2 contained HNA during incubation and NA added after inactivation. Experiment 3 contained both HNA and NA during incubation. Final volume = 3.0 ml, temperature = 25°, incubation time = 1 hour. Reaction started with tipping in of substrates and ended with boiling for 5 minutes.

	Experiment 1		Experiment 2		Experiment 3	
	HNA	NA	HNA	NA	HNA	NA
	c p m per μ mole	c p m per μ mole	c p m per μ mole	c p m per μ mole	c p m per μ mole	c p m per μ mole
Specific activity	1.56×10^5	0	1.31×10^5	5.56×10^2	6.4×10^4	6.3×10^4

tion and elution, NA and HNA of Experiment 3, Table IV, were separated by an IRA-400 column. Fractions 64 to 104 contained NA and Fractions 151 to 175 contained HNA. These were pooled separately, acidified, and steam-distilled. The specific activity of NA from the column was 6.0×10^4 c p m per μ mole and of HNA was 6.1×10^4 c p m per μ mole, values which are in agreement with the results obtained previously.

The reaction discussed may be formulated by the following equation



Extraction of Hydroxylating Enzyme—A water-soluble extract containing the nicotinic acid-hydroxylating enzyme may be prepared by sonic disintegration of the bacteria in 10 volumes of ice-cold 0.03 per cent $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ solution.

These extracts had no activity as measured by the methylene blue reduc-

tion test except when cysteine was present. Studies of the properties and kinetics of the enzymatic reaction are in progress.

DISCUSSION

The present interest in hydroxylation of aromatic and non-aromatic ring compounds stems from the importance of these reactions in the metabolism of such physiologically important compounds as the steroids, tryptophan and tyrosine. In most of these, molecular oxygen seems to be the source of oxygen in the hydroxyl group. This is also true of the hydroxylation of nicotinic acid in *Pseudomonas* as described by Hughes (6). One possible example of anaerobic hydroxylation was indicated by the decolorization of methylene blue by a variety of quinoline derivatives by liver enzymes as reported by Knox (7). Since the bacterial hydroxylation of NA is an example of an anaerobic hydroxylation of an aromatic ring, the source of oxygen of the hydroxyl group is probably water. The mechanism of this reaction no doubt differs from that of the aerobic hydroxylations, although some aspects may be held in common.

The pyridine ring presents certain special problems. It is an aromatic structure having resonance stability of the same order as benzene, however, in terms of reactivity it has been found that the 3, or β , position has typical aromatic properties but that the 2, 4, and 6 positions have anomalous behavior. Because of the electron attraction of the nitrogen atom the electron density in the 2, 4, and 6 positions is reduced. In nicotinic acid the situation is enhanced by the presence of the carboxyl group. Both the nitrogen atom and the carboxyl group increase the reactivity of the 2, 4, and 6 positions with regard to a negatively charged hydroxyl ion.

As an example of this, *N*-methylpyridium hydroxide may be oxidized by potassium ferricyanide to the *N*-methylpyridone. This indicates the addition of the hydroxyl group to the 2 carbon of the ring to form the intermediate *N*-methyl-2-hydroxy-1,2-dihydropyridine and subsequent dehydrogenation to the pyridone (8). The enzymatic reaction may proceed in a similar fashion.

If HNA is an intermediate in the fermentation of NA, it is probable that the cleavage of the ring occurs between the carbon in the 6 position and the nitrogen group. One of the major mechanisms for cleavage of a ring of carbon atoms appears to involve, as a first step, the hydroxylation of adjacent carbon atoms. The formation of the 6-pyridone, however, permits the possibility of a cleavage between the carbon and nitrogen atoms, thus obviating the need for further hydroxylation.

SUMMARY

The anaerobic bacteria which ferment nicotinic acid to acetic and propionic acids, CO_2 , and NH_3 catalyze the reversible anaerobic oxidation of nicotinic acid to 6-hydroxynicotinic acid

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